Characterization of cellular signalling pathways involved in the regulation of trophoblast cell functions

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This dissertation is dedicated to my beloved family
for their endless love, support and encouragement.

วิทยานิพนธ์เล่มนี้ได้ตั้งใจทำเพื่อครอบครัวอันเป็นที่รักของข้าพเจ้า

ขอบคุณสำหรับความรักตลอดมา การสนับสนุน และ กำลังใจที่ยิ่งใหญ่

Esta tesis está dedicada a mi amada familia
por su infinito amor, apoyo y aliento

Diese Dissertation ist meiner geliebten Familie gewidmet
für ihre unendliche Liebe, Unterstützung und Ermutigungen.

„Gedruckt mit Unterstützung des Deutschen Akademischen Austauschdienst“

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List of abbreviations

ActRIIA	Activin Receptor IIA
Ago	Argonaute
ALK5	Activin Receptor-like Kinase 5
ALK7	Activin Receptor-like Kinase 7
BrdU	Bromodeoxyuridine (5-bromo-2'-deoxyuridine)
C19MC	Chromosome 19 microRNA cluster
C14MC	Chromosome 14 microRNA cluster
CAM	Cell adhesion molecule
CC	Cell column
CCND1	Cyclin D1
CCNE1	Cyclin E1
CCNG2	Cyclin G2
CL	Corpus Luteum
CLC	Cardiotrophin-like cytokine
CNTF	Ciliary Neurotrophic Factor
CSE	Cystathionine β-synthase and cystathionine γ-lyase
CT-1	Cardiotrophin-1
CTB	Cytotrophoblast
ECM	Extracellular matrix
Ectb	Endovascular cytotrophoblast
EGF	Epidermal-Growth-Factor
EFNA3	Ephrin-A3
ERK	Extracellular-signal-Regulated Kinases
EVT	Extravillous trophoblast
Exp5	Exportin 5
FGR	Fetal growth restriction
FSH	Follicle stimulating hormone
GAPs	GTP-ase activating proteins
GCM1	Gial cell missing 1
GDM	Gestational diabetes mellitus
gp130	Glycoprotein 130
Grb-2	Growth factor receptor-bound protein 2
HB-EGF	Heparin binding-epidermal growth factor
hCYP191A1	Human aromatase
hES	Human embryonic stem cells
HCG	Human chorion gonadotropin
HGF	Hepatocyte growth factor
HOXA9	Homeobox protein Hox-A9
Hpl	Human placental lactogen
HSD17B1	Hydroxysteroid (17-beta) dehydrogenase 1
ICM	Inner cell mass
Ictb	interstitial cytotrophoblast
IL-11	Interleukin- 11
IL-6	Interleukin- 6
ISCU	Iron-sulfur cluster scaffold
ITGB1	integrin beta 1
IUGR	Intra-uterine growth restriction

JAG1	JAGGED 1
JAK/STAT	Janus kinase/Signal Transducer and Activator of Transcription
JNK	Jun N-terminal kinase
LE	Luminal epithelium
LH	Luteinizing hormone
LIF	Leukemia inhibitory factor
LIFR	Leukemia inhibitory factor receptor
MCL1	Myeloid cell leukemia sequence 1
MEK	Mitogen-activated kinase
MiRNA	MicroRNA
MiRNome	MicroRNA expression signature
MMP-2	Matrix Metalloproteinase-2
MMP-9	Matrix Metalloproteinase-9
MMPs	Matrix Metalloproteinases
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
mTOR	Mammalian target of Rapamycin
NOMO-1	NODAL modulator 1
OSM	Oncostatin M
OSMR	Oncostatin M receptor
PDCD4	Programmed cell death 4
PIAS	Protein inhibitors of activated Stats
PKC	Protein kinase C
PPP3R1	Protein phosphatase 3, regulatory subunit B, alpha
Pre-miRNAs	Precusor microRNAs
Pri-miRNAs	Primary microRNAs
PTEN	Phosphatase and tensin homolog
PTPs	Protein tyrosine phosphatases
qRT-PCR	Quantitative real time polymerase chain reaction
RAS/MAPK	Ras/Mitogen Activated Protein Kinase
RGD	Arginine–glycine–aspartic acid
RKIP	Raf kinase inhibitor protein
RNAi	RNA interference
RISC	RNAi-induced silencing complex
sFlt-1	Soluble fms-like tyrosine kinase-1
SGA	Small for gestational age
SH2	Src homology 2
SOCS	Suppressors of cytokines signalling
SOS	Son of sevenless
sPlGF	Soluble placental growth factor
STB	Syncytiotrophoblast
sVEGF	Soluble vascular endothelial growth factor
TGF-β	Transforming growth factor beta
Tr	Trophoectoderm
U0126	1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene
UTR	Untranslated region
VEGFA	Vascular endothelial growth factor A

Summary

Cytokines and microRNAs (miRNAs) play important roles in pregnancy by regulation of embryo implantation, trophoblast proliferation, invasion and differentiation. Leukemia inhibitory factor (LIF) and Oncostatin M (OSM) are members of the Interleukin (IL)-6 cytokine family and share the gp-130/LIFR receptor. Consequently, LIF and OSM have overlapping biological effects. Signal transduction of LIF and OSM involve mainly the activation of JAK/STAT and MAPK/ERK cascades but their functions in trophoblast cells have not yet been completely investigated and the intracellular mechanisms remain unclear.

miRNAs are molecular regulators of a wide range of biological processes such as proliferation, differentiation, invasion and apoptosis. Altered miRNAs expression has been implicated in several pathologies including cancer, cardiovascular diseases, neurological disorders and diabetes. Furthermore, their dysregulation seems to be connected with the development of pregnancy related diseases.

The aim of this thesis was to elucidate the signaling pathways activated by the cytokines LIF and OSM as well as the role of specific miRNAs in the control of trophoblast cell functions. Several techniques have been applied including Western blotting, zymography, siRNA transfection, qRT-PCR and assays for assessing cell viability, proliferation, migration, invasion and apoptosis.

This publication based doctoral thesis comprises six manuscripts covering the intracellular signaling pathways activated by LIF and OSM and the functional analysis of miR-21 in trophoblastic cells. In the first part, two manuscripts have been included: a review article summarizing the cytokines that regulate trophoblast cell functions and an original research article in which the function and molecular mechanism of LIF and OSM have been investigated. The second part of the thesis comprises four manuscripts starting with an original article in which the miRNA expression profile of trophoblast cell lines was investigated, followed by two review papers describing the current knowledge on miRNAs in pregnancy and finally an original research paper focused on the functional analysis of miR-21 in two trophoblastic cell lines.

The results demonstrate that LIF and OSM have similar functions in activating STAT3 and ERK1/2 by changing their phosphorylation status in four trophoblastic cell lines. However, LIF but not OSM significantly increases invasion of trophoblastic cell lines. Inhibition of STAT3 decreases the invasiveness of trophoblastic cell lines by inhibiting the secretion of matrix metalloproteinases (MMPs). These results suggest that LIF shares STAT3 and ERK1/2 activation with OSM but their resulting responses are cell type dependent.

The second part of this dissertation is focused on placenta-specific miRNAs and their function on trophoblast cells. The miRNA profile of primary cells includes the expression of at three clusters which are specifically expressed in placenta tissue: C19MC, C14MC and the miR-371-3 cluster. C19MC is highly expressed in third trimester trophoblast cells and in choriocarcinoma cell lines. C14MC is highest expressed in first trimester trophoblast cells as well as in HTR-8/SVneo cells and its plasma concentration decreases with the gestational age. Both miRNA clusters are evolutionary related to the appearance of placenta and their dysregulation can be associated with pregnancy pathologies.

Finally, this dissertation covers the functional study of miR-21 in trophoblastic cells. MiR-21 does not belong to C19MC and C14MC members but is one of the highest expressed miRNA in first trimester trophoblast cells and is also highly expressed in the immortalized first trimester trophoblast cell line HTR-8/SVneo and in the analyzed choriocarcinoma cell lines. The results demonstrate that miR-21 regulates trophoblast cell proliferation, migration, invasion and apoptosis by targeting PTEN and PDCD4.

In conclusion, this work describes the intercellular signaling pathways activated by OSM and LIF, as well as the function of miR-21 in trophoblastic cells. This knowledge will help to understand the mechanisms of these molecules in normal placental development, especially in the regulation of trophoblast cell proliferation, migration, invasion and apoptosis. Moreover, it may provide novel information on their involvement in the pathogenesis of pregnancy-related disorders which may be useful for the design of future non-invasive diagnostic tools or for the development of new therapeutic approaches.

Zusammenfassung

Zytokine und microRNAs (miRNAs) spielen eine wichtige Rolle während der Schwangerschaft durch die Regulation der Embryo-Implantation, der Trophoblast-Proliferation, -Invasion und –Differenzierung. Leukemia Inhibitory Factor (LIF) und Oncostatin M (OSM) sind Mitglieder der Interleukin (IL)-6 Zytokin-Familie und teilen sich den gp-130/LIFR Rezeptor. Folglich haben LIF und OSM überlapende biologische Effekte. Die Signaltransduktion von LIF und OSM schließt die Aktivierung der JAK/STAT und MAPK/ERK Kaskaden ein, aber die resultierenden Funktionen in Trophoblastzellen sind noch nicht vollständig untersucht und zahlreiche intrazelluläre Mechanismen noch weitgehend unbekannt.

MiRNAs sind molekulare Regulatoren einer großen Zahl biologischer Prozesse, so wie Proliferation, Differenzierung, Invasion und Apoptose. Veränderte miRNA-Expression ist involviert in zahlreiche Pathologien einschließlich Malignome, kardiovaskuläre Erkrankungen, neurologische Störungen oder Diabetes. Darüber hinaus erscheint ihre Fehlregulation auch mit der Entwicklung von Schwangerschaftserkrankungen zusammenzuhängen. Eine wachsende Zahl von Studien wurde in der letzten Zeit publiziert, die miRNAs eine entscheidende Rolle bei der Embryoimplantation und der Placentaentwicklung sowie ihren Störungen beimessen, aber die Kenntnisse sind noch begrenzt.

Das Ziel dieser Arbeit war, die intrazellulären Signalwege, die durch LIF und OSM aktiviert werden, sowie die Rolle spezifischer miRNA in der Kontrolle von Trophoblastzellfunktionen zu untersuchen. Verschiedene Methoden wurden angewendet, wie z.B. Western blotting, Zymographie, siRNA Transfektion, qRT-PCR sowie Assays zur Messung der Zell-Viabilität, -Proliferation, -Migration, -Invasion und Apoptose.

Diese publikationsbasierte Dissertation beinhaltet sechs Manuskripte, die LIF- und OSM-aktivierte intrazelluläre Signalwege untersuchen und Funktionen von miR-21 in Trophoblastzellen analysieren. Der erste Teil schließt zwei Manuskripte ein: einen Review-Artikel, der die Rolle von Zytokinen beschreibt, die Trophoblastzell-Funktionen regulieren sowie eine Original-Arbeit, in der die Funktionen und

molekularen Mechanismen von LIF und OSM untersucht wurden. Der zweite Teil der Arbeit beinhaltet vier Manuskripte beginnend mit einem Original-Artikel, in dem die miRNA Expression-Profile von Trophoblastzellen und -linien untersucht wurden, gefolgt von zwei Review-Artikeln, die den aktuellen Wissensstand zu miRNAs in der Schwangerschaft zusammenfassen, sowie zu letzt eine Original-Arbeit, die funktionelle Analysen zu miR-21 in zwei Trophoblastzelllinien beschreibt.

Die Ergebnisse zeigen, dass LIF und OSM ähnliche Eigenschaften bezüglich der Phosphorylierung von STAT3 und ERK1/2 in vier trophoblastären Zelllinien haben. LIF, aber nicht OSM steigert die Invasion trophoblastärer Zelllinien signifikant. Die Inhibierung von STAT3 reduziert die Invasivität von Trophoblastzelllinien durch Blockierung der Sekretion von Matrix-Metalloproteinasen (MMPs). Diese Ergebnisse suggerieren, dass LIF wie OSM STAT3 und ERK1/2 aktiviert, aber dass die funktionellen Antworten Zelltyp-abhängig sind.

Der zweite Teil dieser Dissertation ist auf Placenta-spezifische miRNAs und ihre Funktionen in Trophoblastzellen fokussiert. Es konnte gezeigt werden, dass miRNA-Profile von primären Trophoblastzellen die Expression von drei Clustern einschließen, die spezifisch in Plazenta-Gewebe exprimiert werden: C19MC, C14MC und das miR-371-3 Cluster. C19MC ist stark exprimiert in Trophoblastzellen des dritten Trimester und in den Chorioncarcinomzelllinien. C14MC ist am stärksten in Ersttrimester Trophoblastzellen und in HTR-8/SVneo Zellen exprimiert. Die Konzentration von C14MC miRNAs fällt mit zunehmendem Schwangerschaftsalter. Beide miRNA Cluster sind evolutionär mit dem Erscheinen der Placenta verbunden. Ihre Fehlregulation kann mit Schwangerschaftspathologien in Zusammenhang stehen.

Schließlich wurden in dieser Arbeit funktionelle Studien zu miR-21 in trophoblastären Zellen durchgeführt. MiR-21 gehört nicht zu den C19MC- und C14MC-Clustern, ist jedoch eine der am stärksten exprimierten miRNA in Ersttrimester-Trophoblastzellen und hoch exprimiert in der immortalisierten Ersttrimester-Trophoblastzelllinie HTR8/SVneo sowie auch in Chorioncarcinomzelllinien. Die Ergebnisse zeigen, dass miR-21 die Trophoblastzell-Proliferation, -Migration, -Invasion und -Apoptose durch Regulation von PTEN und PDCD4 beeinflusst.

Zusammengefasst beschreibt diese Arbeit die intrazellulären durch OSM and LIF aktivierten Signalwege sowie die Funktionen von miR-21 in Trophoblastzellen. Diese Kenntnisse werden dazu beitragen, die Funktionen dieser Moleküle in der Placentaentwicklung besser zu verstehen. Darüber hinaus, liefert sie Informationen über deren mögliche Beteiligung an der Pathogenese von Schwangerschaftsstörungen, was von Nutzen für die Entwicklung zukünftiger nicht-invasiver Diagnosemethoden oder therapeutischer Ansätze sein kann.

Chapter 1 Introduction

1.1 Origin of human life

Fertilization is the interaction process between spermatozoa and egg to form the zygote. The zygote develops into the blastocyst which attaches to maternal endometrium by a serie of processes that occur from the fallopian tube to the uterine cavity (Paiva et al. 2009). In a normal human menstrual cycle (28-30 d), the rising levels of estrogen control the growth of ovarian follicles and restore the endometrium (Norwitz et al. 2001) (Fig.1). During proliferative phase, pituitary gonadotropin stimulates folliculogenesis and allowing the selection of the dominant follicle. At day 14 (midcycle), follicle stimulating hormone (FSH) and luteinizing hormone (LH) are highest levels leading to ovulation.

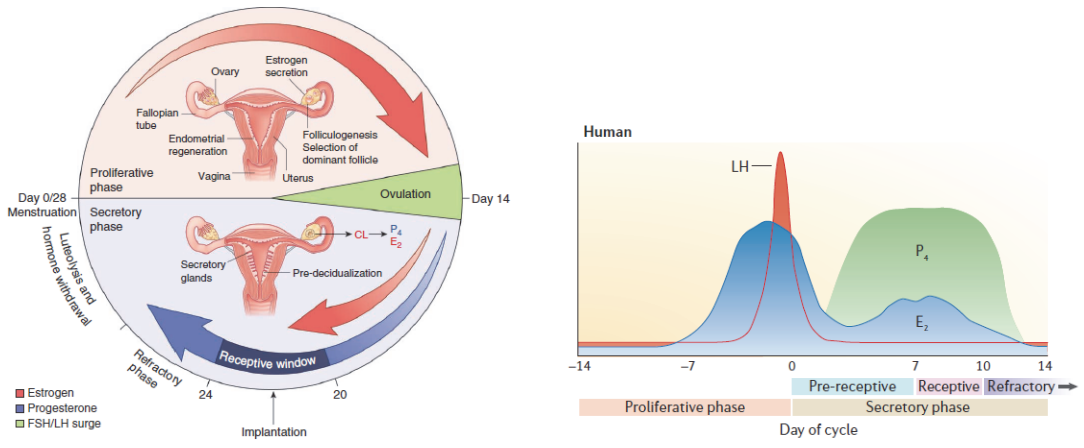

Figure 1. Window of uterine receptivity and hormones during implantation. Based on histological and functional of human uterine, the uterine is classified into proliferative (follicular) phase and secretory (luteal) phase during 28-30 day of menstrual cycle. During the midluteal phase (cycle day 20-24), is defined as window of implantation, which conducting to implantation and subsequent pregnancy. During the secretory phase, the uterus is considered pre-receptive for 7 days following ovulation (day 0) or around day 21 of menstrual cycle. Subsequently, the uterine turns into receptive (7-10 days after ovulation) and refractory (11-14 days after ovulation), respectively. Adapted from Wang and Dey and Cha *et al* (Wang und Dey 2006, Cha et al. 2012)

The remains of follicle become a corpus luteum (CL), whose main function is the production of progesterone (P4). Progesterone influences thickening of the endometrium preparing it for implantation (Norwitz et al. 2001). During the mid of secretory phase, the window of receptivity (window of implantation) occurs, in this period of time the blastocyst can implant resulting in pregnancy establishment (Fig. 2). In the absence of embryo, the corpus luteum involutes dropping the levels of progesterone and estrogen, and resulting in menstruation (Wang und Dey 2006).

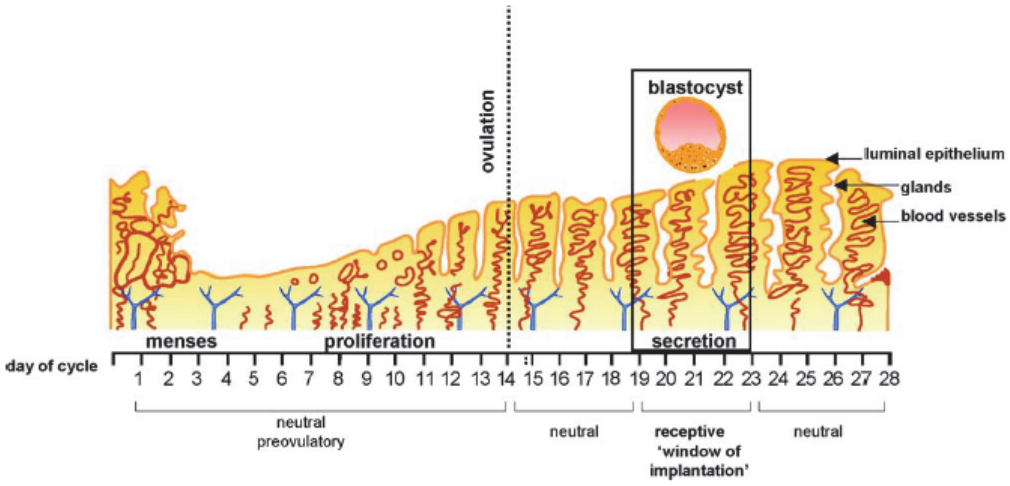

Figure 2. The implantation of blastocyte into the endometrium. The endometrium can maintain blastocyte implantation only during the window of implantation (day 6-9 following the luteinizing hormone (LH) peak). Taken from Paiva *et al* (Paiva et al. 2009)

1.2 Implantation and placental development

3-4 days after fertilization, a spherical shape of dividing embryo cells becomes a compact mass of cells known as “morula” (16-32 cells stage). By day 4-5, the embryo forms a central fluid-filled cavity which is referred to as a “blastocyst”. The mature blastocyst is an assembling of 3 cell types of layers: the outer epithelial trophectoderm (Tr), the primitive endoderm (PE) and the pluripotent inner cell mass (ICM). On day 6 (blastocyst stage), the blastocyst escapes from its outer layer, zona pellucida, and implants itself in the uterine wall (Figure 3) (Wang und Dey 2006).

The aims during implantation are to invade the maternal uterine tissue and to obtain major nutrients for the embryo development (van Mourik et al. 2009).

The process of implantation comprises distinct stages which initiate with the “apposition”. During this stage, the blastocyst becomes closely and interacts with the luminal epithelial (LE) of the endometrium. In the second stage, “adhesion”, the blastocyst attaches to the LE in a process controlled by adhesion molecules, immune cells and cytokines. The third stage is trophoblast invasion, this step allows trophoblast cells to penetrate through LE and basal lamina of the stroma, reaching as far as the maternal vasculature (Figure 3) (van Mourik et al. 2009, Cha et al. 2012, Achache und Revel 2006, Fitzgerald et al. 2008).

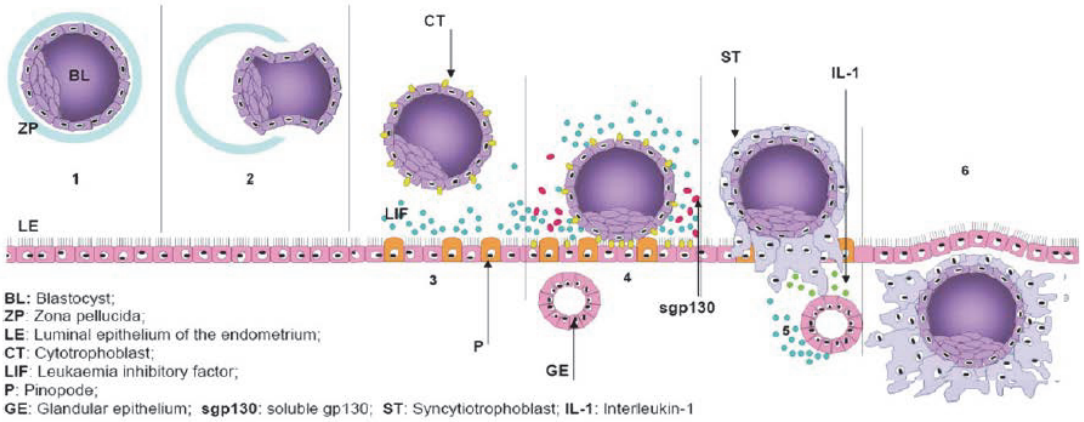

Figure 3. Mechanism of blastocyte implantation. (1) Free floating blastocyte is encased by zona pellucida. (2) Blastocyst escapes from zona pellucida. (3) Apposition stage, endometrium expresses LIF and blastocyst expresses LIF receptors. Pinopodes appear on the endometrial surface. (4) The blastocyst adheres to the endometrium and subsequently produces LIF itself, while the LIF receptor and gp130 on endometrium increases. (5) Interaction between blastocyte and endometrium induces trophoblast cells differentiate into inner cytotrophoblast and outer syncytiotrophoblast. Trophoblast cells invade into the luminal epithelium and initiate itself to produce IL-1. (6) The implantation process is complete. Taken from Fitzgerald *et al* (Fitzgerald et al. 2008)

Placentation begins when the blastocyst makes contact with the uterus shortly after implantation. At day 5 after fertilization, the outer membrane (chorion) and the trophoblast layer of placenta are derived from the trophoectoderm of the blastocyst. The chorion develops chorionic villi and the mesoderm in the core of villi form

vessels, which can be extend into the embryo as umbilical arteries and veins. At day 12-15, trophoblast layer forms the primary villi consisting of column cytotrophoblast surround by syncytiotrophoblasts. Around day 15-21, extra-embryonic mesenchymes penetrate into trophoblast cells column which generates the secondary villi. After day 18 of fertilization, tertiary villi is formed by vasculogenesis inside of placental villi (Chaddha et al. 2004). As the embryo grows and expands, it pushes the chorionic villi furthest away leaving functioning chorionic villi and maternal blood on only one side of the embryo. This structure is called the placenta. The placenta works as an interface between the mother and fetus and regulates the exchange of gases, nutrients and waste products (Lewis et al. 2012). The word "*placenta*" is derived from Greek word *"plakoenta*", which means flat cake because of the flat round shape (Rossant und Cross 2001).

1.3 Trophoblast invasion

Attachment of the embryo to the endometrium triggers trophoblast differentiation into the outer syncytiotrophoblast (STB) and the inner cytotrophoblast (CTB) (Suman et al. 2013). The fusion of CTB generates the non-proliferative multinucleated syncytium (villous pathway) which is referred to as STB. The STBs cover the entire surface of the floating villi and are responsible for gases and protein transport, nutrient supply, waste elimination and production of several hormones including human chorionic gonadotrophin (HCG), estrogen, progesterone, and human placental lactogen (hPL) (Lunghi et al. 2007). The formation of floating and anchoring villi begins immediately after nidation (Lunghi et al. 2007). Once the anchoring villi are attached to the decidua, mononuclear CTBs form the multilayered trophoblast cell column (CC) through proliferation. Trophoblast cells in the column at the tip of anchoring villi differentiate into extravillious trophoblast (EVT) (extravillous pathway) (Knofler 2010). The invasive EVT can be classified in 2 types: the interstitial EVT which invade maternal decidual stroma as far as the inner third of the myometrium (Wallace et al. 2012). EVTs move towards the spiral arteries of the decidua and differentiate into giant multinuclear cells (Anin et al. 2004). The second EVT type is the endovascular trophoblast, which plays an important role in remodeling maternal

spiral arteries. The endovascular trophoblast cells express an endothelial phenotype and typical vascular adhesion molecules (Lyall et al. 2001). The endovascular trophoblast invades the lumen of the arteries by replacement the vascular smooth muscle and endothelium of the vessels, which resulting in enlargement of the vessel diameter (Figure 4.) (Lunghi et al. 2007, Knofler 2010, Cartwright et al. 2010).

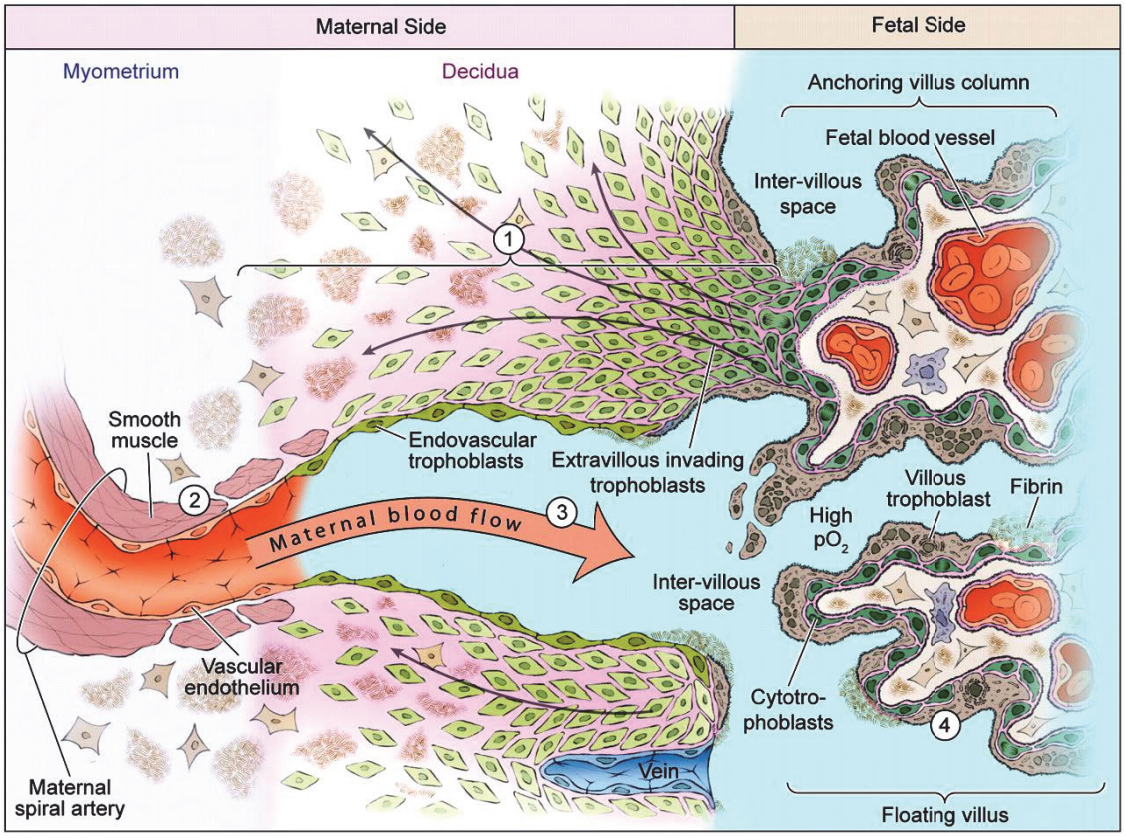

Figure 4. Trophoblast invasion into maternal decidua. 1) The proliferative column trophoblast cells are formed and differentiated into invasive extravillious trophoblast subtypes; interstitial cytotrophoblasts and endovascular cytotrophoblasts. The interstitial trophoblasts migrate into maternal decidua stroma and form giant cells at the end stage. 2) Interstitial trophoblasts migrate into spiral arteries in order to colonize the vascular lumen by the formation of endovascular trophoblasts. 3) These changes facilitate high volume of blood flow into intervillous space. 4) Syncytiotrophoblast is direct contact with maternal blood. Taken from Kingdom *et al.*(Kingdom und Drewlo 2011)

The vascular remodeling allows maternal blood circulation in low resistance vessels. Failure in EVT invasion and vascular remodeling is associated with pregnancy complications such as preeclampsia and intra-uterine growth restriction (IUGR) (Knofler und Pollheimer 2013, Pijnenborg et al. 1991, Kaufmann et al. 2003, Zhou et al. 2013).

1.4 Preeclampsia

Preeclampsia (PE) is defined by new-onset hypertension and proteinuria at ≥ 20 weeks of gestation and affects 3-5 % of pregnancy worldwide. It is considered the major cause of maternal morbidity and is associated with fetal pathologies including preterm birth, small for gestational age (SGA), intra-uterine growth restriction (IUGR) and can resulted in fetal death (Fu et al. 2013a, Chaiworapongsa et al. 2014). The molecular mechanism of PE is largely unknown. Various experiments have been demonstrated that PE is associated with ischemia, hypoxia, angiogenic proteins such as soluble fms-like tyrosine kinase-1 (sFlt-1), soluble vascular endothelial growth factor (sVEGF) and soluble placental growth factor (sPlGF) (Mutter und Karumanchi 2008, Chaiworapongsa et al. 2014). The spiral artery remodeling is a key step in placental development resulting in vessels with increase diameter, decrease resistance and increase blood flow. Impaired remodeling of spiral artery by cytotrophoblast cell invasion has been especially focused on PE (Crosley et al. 2013) (Figure 5).

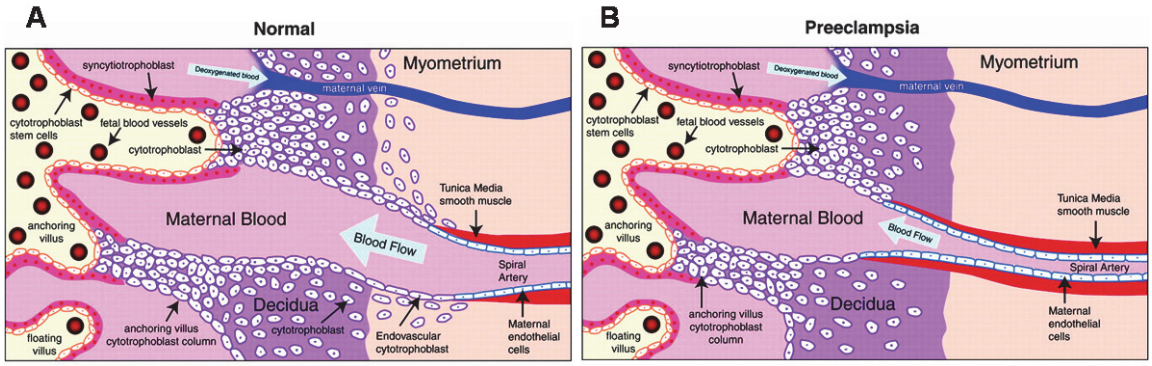

Figure 5. Pathophysiology placental development in preeclapmsia. A) extravillous trophoblast cells invade into one-third of the myometrium. The endovascular extravillous trophoblast replace endothelial layer of maternal spiral artery and increase vessel diameter, decrease resistance and increase blood flow. B) Spiral artery is lack of cytotrophoblast invasion into myometrial segment resulting in narrow of spiral artery, decrease blood flow and reduce uteroplacental perfusion. Taken from Kim *et al* (Kim 2013)

1.5 Key regulatory network in implantation and placental development

Normal conception in human per cycle is poor (~30%), and 75% of lost pregnancies are caused by implantation failure (Norwitz et al. 2001). Therefore, blastocyst implantation is a crucial step for placental development and establish of pregnancy. Cell differentiation, proliferation, migration, invasion, angiogenesis and apoptosis also play a role during placental development (Guzeloglu-Kayisli et al. 2009, Fu et al. 2013a). All these processes are temporally and spatially regulate throughout pregnancy (Fu et al. 2013a). A large number of mediators have been proposed to regulate placental development including adhesion molecules, growth factor, hormones, cytokines and miRNAs (McEwan et al. 2009, Galliano und Pellicer 2014, Fu et al. 2013a).

1.5.1 Cell adhesion molecules

Cell adhesion molecules (CAM) are glycoproteins expressed on cell surface that play important roles in the maintenance of tissue integration, wound healing, morphogenic movement, cellular migrations and tumor metastasis (McEwan et al. 2009). They are consist of four groups: integrins, cadherins, selectins and immunoglobulins (McEwan et al. 2009). During the pre-implantation stage, blastocyst expresses integrins and that are likely to control the cleavage and development of blastocyst as well as the initial adherence to the uterine luminal epithelium (Guzeloglu-Kayisli et al. 2007). Among integrins, β3 subunit is highly expressed during the mid-secretory phase by the luminal and glandular epithelium, and functions in the regulation of endometrial receptivity (Zhang et al. 2013). Cytotrophoblast cells express integrins, which support the attach to the extracellular matrix (ECM) and regulate their invasion (van den Brule et al. 2005). Prior to implantation, E-cadherins are highly expressed in the luminal epithelium but their expression is down-regulated before blastocyst invasion. Embryos lacking E-cadherin gene, failed to establish adhesion junctions in the trophectoderm and die in the pre-implantation period. This suggests that remodeling of E-cadherins between epithelium is an important process for implantation (Zhang et al. 2013). Improvement

of implantation is associated with high expression of L-selectin ligands which are immunolocalized to the luminal and gland epithelial (Wang et al. 2008, Hey und Aplin 1996, Singh und Aplin 2009). CD146 is a member of immunoglobulin superfamily and it is expressed only in invasive cytotrophoblasts, suggesting that CD146 may play an important function in trophoblast invasion (Liu et al. 2004).

1.5.2 Growth factors

Growth factors comprise a family of secreted signaling molecules capable of inducing proliferation and differentiation in mammalian cells (Guzeloglu-Kayisli et al. 2009). These factors bind to specific cell surface receptors, which include a tyrosine kinase domain in their C terminus. Upon ligand binding, the receptors further initiate signaling cascades by phosphorylation of various molecules (Singh et al. 2011). The expression of several growth factors and their receptors in the uterus is cell specific and temporal during the pre-implantation phase, suggesting that some of these factors are important for the blastocyst implantation (Tazuke und Giudice 1996). The presence of epidermal growth factor (EGF) in human endometrium, placenta, and trophoblast cells, upon localization to stromal cells in secretory endometrium suggests its functions on blastocyst implantation (Tazuke und Giudice 1996, Guzeloglu-Kayisli et al. 2009, Hofmann et al. 1991). Transforming growth factor beta (TGF-β) is localized in human endometrial stromal, epithelial, decidual cells and is involved in trophoblast adhesion to the endometrium during implantation (Bischof und Campana 2000, Feinberg et al. 1994).

1.5.3 Hormones

Progesterone and estrogen are the major steroid hormones that are essential for uterine receptivity and facilitate blastocyst implantation (Wang und Dey 2006). In proliferative phase, estrogen plays a role in preparing the endometrium for progesterone action as described previously (Fig.1). Additionally, Progesterone is essential for implantation and pregnancy maintenance in all mammals, whereas the requirement for estrogen is species-specific (Dey et al. 2004). Progesterone plays a role in proliferation, differentiation and maintenance of endometrial stromal, glandular and myometrium cells (Huet-Hudson und Dey 1990).

1.5.4 Interleukin-6 family of cytokines

Interleukin-6 (IL-6) family comprises IL-6, IL-11, LIF (leukemia inhibitory factor), OSM (oncostatin M), CNTF (ciliary neurotrophic factor), CT-1 (cardiotrophin-1) and CLC (cardiotrophin-like cytokine) (Heinrich et al. 2003). IL-6 family is involved in immune responses, cell survival, apoptosis, proliferation and invasion (Eulenfeld et al. 2012, Fitzgerald et al. 2005, Fitzgerald et al. 2011). In addition, it is well known that cytokines IL-6 family is essential during blastocyst implantation (Dimitriadis et al. 2005). Among these cytokines, LIF is crucial for uterine receptivity and implantation, as its deletion results in implantation failure in mice (Stewart et al. 1992). Cytokines of the IL-6 family share transmembrane protein gp130 (glycoprotein 130) to form receptor complexes specific for each cytokine (Figure 6).For instance, LIF induces heterodimertization of gp130 and the leukemia inhibitory factor receptor (LIFR), while OSM triggers the heterodimerization of the gp130 with LIFR or the specific oncostatin M receptor (OSMR) (Fig 6) After cytokine recognition by the receptors, several intracellular pathways became activated including the Janus kinase/Signal Transducer and Activator of Transcription (JAK-STAT) pathway by the activation of STAT3 (Yun et al. 2012).

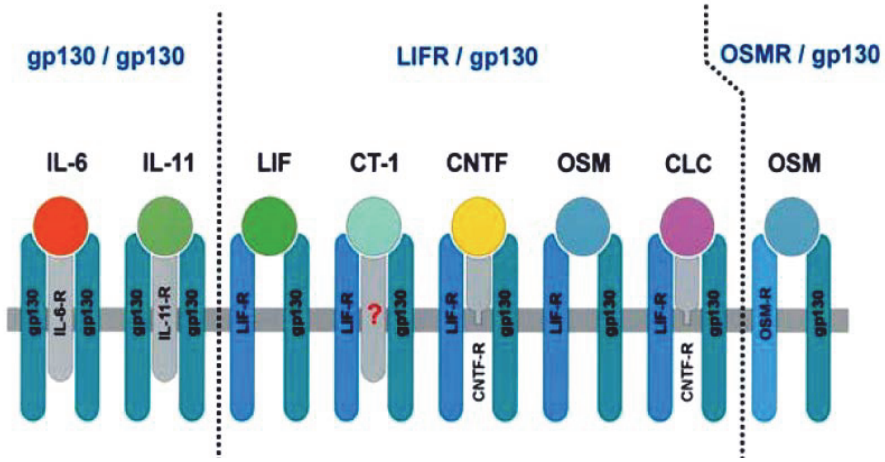

Figure 6. Difference receptors of IL-6 cytokine family. IL-6 type cytokine receptor complexes signal through different combination between LIFR, OSMR and gp130. LIF triggers the signaling pathway through heterodimer receptor of LIFR/gp130, while OSM can bind both LIFR/gp130 and OSMR/gp130 heterodimers. Taken from Heinrich *et al* (Heinrich et al. 2003)

1.6 JAK-STAT and MAPK signaling pathways

The JAK-STAT pathway mediates signals from the cytokine receptors at the plasma membrane to the cell nucleus (Kiu und Nicholson 2012). Upon ligand binding, cytokines receptors dimerize causing receptor activation and transphosphorylation of the receptor-associated JAK molecules (Kiu und Nicholson 2012). These activated JAKs can phosphorylate tyrosine residues in the cytoplasmic region of the receptors to create docking sites for members of the Signal Transducers and Activators of Transcription (STATs) family (Mohr et al. 2012). This leads to tyrosine phosphorylation of STATs. Tyrosine phosphorylated STATs are release from the receptor, thereafter, they dimerize and translocate into the nucleus where they bind specifically to sequences within the promoter regions of DNA inducing the transcription of target genes (Fig.7) (Kiu und Nicholson 2012, Mohr et al. 2012).

Among STATs, STAT3 is the main transcription factor that gets activated by IL-6 type cytokines (Yu et al. 2009, Santos und Costa-Pereira 2011). It is importance in the JAK/STAT pathway in embryo implantation has been showed by the embryonic lethality of STAT3-deficient mice (Takeda et al. 1997).

The JAK/STAT cascade requires a tight cellular regulation to control the magnitude and duration of signaling (Kiu und Nicholson 2012). Moreover, negative regulation prevents inappropriate activities that are associated with disease progression. There are three major specific mechanisms for negative regulation:

1) Tyrosine phosphatases that can dephosphorylate of JAKs in membrane-associated receptor complex or by phosphotyrosine phosphatases (PTPs) in the nucleus.

2) Direct inhibition of phosphorylated STAT dimmers, which prevent DNA recognition by protein inhibitors of STATs (PIAS).

3) Suppressor of cytokine signalling (SOCS) proteins, which direct inhibit JAK enzymatic activity, leading to block JAK and STATs activation. (Fig.7) (Kiu und Nicholson 2012, Croker et al. 2008, Aaronson und Horvath 2002)

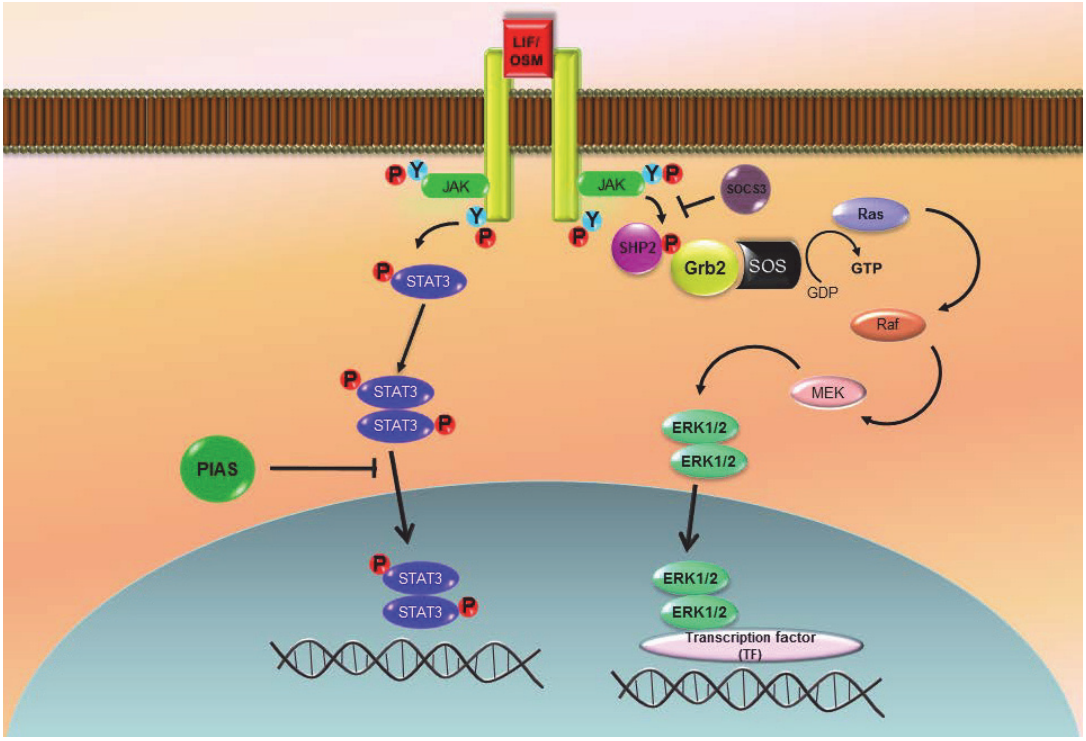

Figure 7. Proposed scheme of LIF and OSM signaling pathway in trophoblast cells. LIF and OSM binding to the receptors and activation of JAK/STAT and MAPK cascades independently. Designed by Chaiwangyen.

Besides activation of STAT transcription factors by IL-6 cytokine family, these cytokines can also trigger activation of the mitogen activated protein kinase (MAPK) cascade (Ramos 2008). IL-6 cytokines bind to their receptors which subsequently

autophosphorylate on tyrosine residues in their cytoplasmic tails (Wortzel und Seger 2011). These phosphorylated residues serve as binding sites for proteins that contain a Src homology 2 (SH2) domain which leads to the recruitment of growth factor receptor-bound protein 2 (Grb-2), which in turn binds the guanine exchange factor son of sevenless (SOS) (Ramos 2008). Recruitment of SOS to the membrane promotes its interaction with the membrane localized small GTPase Ras and results in GTP loading and activation of Ras. This is followed by the sequential recruitment of the MAP kinase kinase kinase Raf to the membrane for subsequent activation by phosphorylation. Activated Raf isoforms phosphorylate and activate the MAP kinase kinases MEK1/MEK2, which in turn activate the effector MAP kinases ERK1 and ERK2 by phosphorylation of the Thr and Tyr residues within their activation loop (Meloche und Pouyssegur 2007, Omerovic et al. 2007, Ramos 2008, Roskoski 2012). Once activated, ERK1/2 are translocated into the nucleus, where they phosphorylate and activate transcription factors. Activated ERK can negatively regulate ERK pathway by inactivation of the Ras activating exchange factor complex Grb2-SOS (Newlands und Houslay 1991). Raf kinase inhibitor protein (RKIP) and Sprouty are also endogenous negative regulators in ERK signaling transduction cascade. RKIP inhibits Raf kinase activity while, Sprouty blocks Ras activation by binding to Grb-2 (Ramos 2008) (Figure 7).

The JAK-STAT pathway is critical for cell proliferation, cell differentiation, cell migration, cell apoptosis and homeostasis (Aaronson und Horvath 2002). Likewise, ERK cascade is also involved in the regulation of a variety cellular processes including cell adhesion, cell cycle progression, cell migration, cell differentiation and cell proliferation (Roskoski 2012). As described previously, LIF and OSM share the cellular receptor resulting in similar functions. Therefore, in the second paper of this work, JAK-STAT and ERK cascades will be analyzed upon LIF and OSM stimulation in four trophoblastic cell lines: the immortalized human trophoblast HTR-8/SVneo, the choriocarcinoma JEG-3, and the hybrids of choriocarcinoma with first and third trimester trophoblast AC1-M59 and ACH-3P cells, respectively. The effects of LIF and OSM will be compared with particular regard to intracellular mechanisms, including STAT3 phosphorylation, STAT3-DNA binding activity, viability and invasion assays.

1.7 microRNAs (miRNAs): tiny molecules but important functions in reproductive biology research

1.7.1 miRNA discovery and biogenesis

The biological discovery of miRNAs was exceptionally beneficial to the research of modern molecular biology. The first miRNA, lin-4, was discovered in 1993 by Lee, Feinbaum, and Ambros in *Caenorhabditis elegans*. This miRNA contained sequences complementary to the 3′ untranslated region (UTR) of the lin-14 mRNA, which was known to encode a developmentally important temporal control protein (Lee et al. 1993, Wightman et al. 1993). Thousands of miRNAs have been discovered since then representing one of the greatest outbreaks in the story of molecular biology.

microRNAs (miRNAs) are short (~22-25 nucleotides) non-coding single stranded RNA molecules that play important regulatory roles by targeting mRNAs for cleavage or translational repression (Finnegan und Pasquinelli 2013). miRNAs are transcribed in the nuceli mainly by RNA polymerase II as primary miRNA (pri-miRNA) (Ha und Kim 2014). Pri-miRNAs are cleaved into stem-loop structure of approximately 70 nucleotide (nt) called precursor miRNAs (pre-miRNAs) by the nuclear RNase III Drosha present in microprocessor complex, which contains the double stranded RNA binding protein, DGCR8 (Graves und Zeng 2012). The pre-miRNA often contains a 2-nt overhang at the 3´ end, as a result of Drosha's RNase III activity and can be exported to the cytoplasm by Exportin 5 (Exp5) associated with its Ran cofactor coupled to GTP (Graves und Zeng 2012). In the cytosol, the pre-miRNA is further processed by the RNA III enzyme Dicer, which binds to 3' overhang of the pre-miRNA and generates a 22 bp double-strand RNA or mature miRNA (Morales Prieto und Markert 2011). One strand of the miRNA (called "guide strand" or mature miRNA) is then coupled to the ribonucleoprotein complex known as RISC (RNA-Induced Silencing Complex) comprising also Dicer, TRBP, PACT and Argonaute (Ago) proteins (Graves und Zeng 2012, Morales Prieto und Markert 2011). The other strand (called "passenger strand" or the star (*)-strand) is degraded (Shukla et al. 2011, Treiber et al. 2012). MiRNAs recognize their targets through pairing of their

seed sequence, which locate in its 5´ end and expand from nucleotide 2-7(Fig. 8) (Graves und Zeng 2012, Shukla et al. 2011, Treiber et al. 2012).

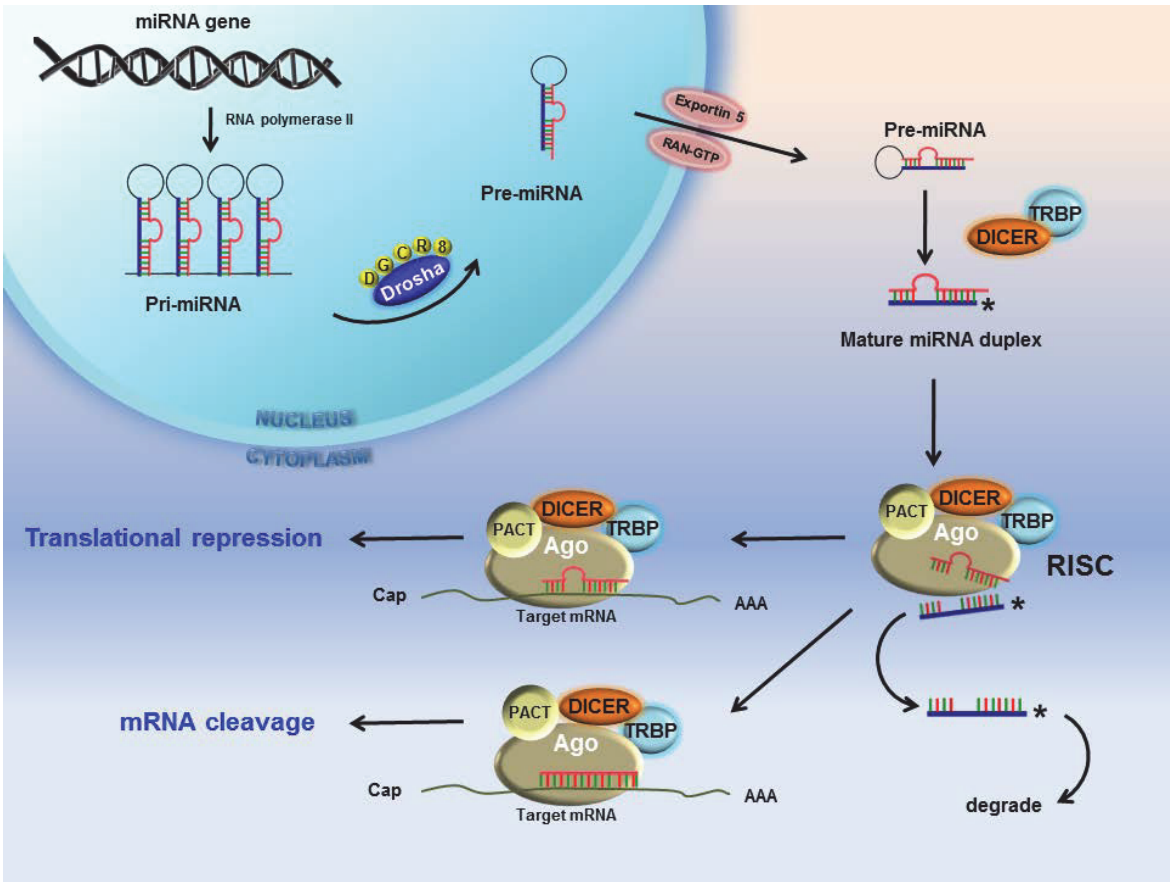

Figure 8. Biogenesis of miRNA. MiRNAs are transcribed by RNA polymerase II in the nucleus and generate pri-miRNA. Microprocessor complex generate a stem-loop nucleotide as pre-miRNA, which is exported to the cytoplasm by exportin 5 and Ran-GTP. Dicer then processes the pre-miRNA into mature miRNA duplex. MiRNA duplexes form complex with RNA induced silencing complex (RISC), leading to unwinding of duplex to form single stranded mature miRNA. The miRNA-RISC complex can bind to the target mRNA through partial sequence complementarity or perfect complementarity, causing inhibition of protein translation and/or degradation of mRNA. Designed by Chaiwangyen

1.7.2 miRNA nomenclature

miRNAs are assigned unique numerical identifiers. Novel miRNAs are experimentally validated and submitted to miRBase data base (http://www.mirbase.org/) for allocation of names before the first publication in peer-reviewed journals (Griffiths-Jones et al. 2006, Arora et al. 2013). The general miRNA nomenclature features the following rules (Griffiths-Jones et al. 2008, Arora et al. 2013):

1. The mature miRNAs are designated by “miR”, while “mir” refers to pre-miRNAs or pri-miRNAs and “*MIR*” refers to gene encoding miRNA.
2. The miRNA name contains a three or four letter species prefix and a numeric suffix (e.g. Homo sapiens = hsa-mir-141, Mus muculus = mmu-mir-192).
3. A mature miRNA sequence may be predicted to be expressed from more than one hairpin precursor locus, designated with further numeric suffixes (e.g. hsa-mir-101-1 and hsa-mir-101-2).
4. Related hairpin loci expressing related mature miRNA sequences have lettered suffixes (e.g. hsa-mir-519a, hsa-mir-519b and hsa-mir-519d).
5. miRNAs that sequences originating from opposite arms of the same hairpin precursor are designated with * or -3p suffix for 3’ end and -5p suffix for 5’ end (e.g. hsa-mir-21*/hsa-mir-21 or hsa-mir-21-3p/ hsa-mir-21-5p)

1.7.3 miRNA targets and mechanism of action

miRNAs bind to their target mRNA resulting in the regulation of gene expression. The most regular interaction of miRNA:mRNA is based on Watson-Crick (WC) pairing between the 5’ end of the miRNA (seed sequence) and the miRNA recognition elements (MREs) usually located on the 3’ UTR of the mRNA targets (Chang und Mendell 2007, Arora et al. 2013, Felekkis et al. 2010). The seed sequence encloses 2-8 nucleotides (Peterson et al. 2014). Perfect complementary of seed sequence and target mRNA allows Ago-catalyzed cleavage of the mRNA strand, while incomplete binding lead to repress of mRNA translation (Fig. 8)(Arora et al. 2013). There are several types of seed matches depending on algorithm as follows (Fig.9) (Peterson et al. 2014):

1. 8mer: a perfect WC match from nucleotides 2-8 of the miRNA seed in addition to an A across from the nucleotide 1
2. 7mer-A1: a perfect WC match from nucleotides 2-7 of the miRNA seed in addition to an A across from the nucleotide 1
3. 7mer-m8: a perfect WC match from nucleotides 2-8 of the miRNA seed
4. 6mer: a perfect WC match between the miRNA seed and mRNA for 6 nucleotides

Generally, one miRNA can regulate hundreds of different target genes and one gene can be regulated by hundreds of miRNAs, leading in tremendous regulatory potential (Felekkis et al. 2010).

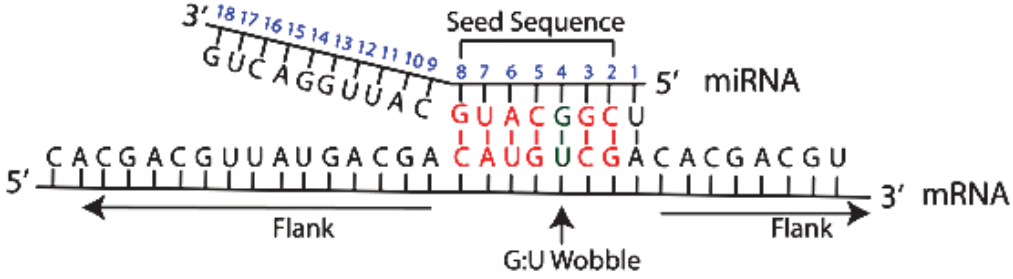

Figure 9. Schematic of miRNA seed sequence. Nucleotide position of miRNA is shown in blue and seed sequence of miRNA refers to nucleotide position 2-8. MiRNA-mRNA pairings are shown in red and mismatch (G:U Wobble) of WC is shown in green. Franks refer to the mRNA sequence on either side of the region corresponding to the miRNA seed sequence. Taken from Peterson *et al (Peterson et al. 2014).*

1.7.4 Biological functions of miRNAs

To date, more than 25,000 mature miRNAs in 193 species and more than 2000 miRNAs in the human genome have been analyzed according to miRbase database release 20 (Liang et al. 2013, Li et al. 2014b, Griffiths-Jones et al. 2008). MiRNAs play an important role in many different biological processes including cell differentiation, cell proliferation and apoptosis (Huang et al. 2011). Dysfunctions of miRNAs could be associated with a number of human diseases including cardiovascular diseases, neurological disorders and cancer (Finnegan und Pasquinelli 2013).

1.7.4.1 miRNAs in development

Two miRNAs, lin-4 and let-7 have been associated with larva development in *C. elegans* as earliest miRNAs discovery (Lee et al. 1993, Reinhart et al. 2000). Recently, miR-1, miR-133a, miR-133b, miR-206, miR-208, miR-208b, miR-486 and miR-499 have been elucidated in cardiac and/or skeletal muscle development (referred to as myomiRs)(Guller und Russell 2010). MiR-1 is highly expressed in cardiac myocyte and involved in myocyte differentiation and development of heart, while miR-133 increases myocyte proliferation (Zhao et al. 2007, Guller und Russell 2010). Moreover, miR126, miR-143, miR-145 and miR-218 have been involved in vascular and blood development (Small und Olson 2011). Specific miRNAs in mammalian nervous system, miR-9 and miR-124, are implicated in neuronal development including early neurogenesis, neuronal differentiation, dendritic morphogenesis and synaptic plasticity (Gao 2010). Several miRNAs have been reported to regulate hematopoiesis and immune system including miR-17~92, miR-21, miR-144, miR-146 miR-150, miR-181, miR-196b, miR-221, miR-222, miR-223, miR-451(Sayed und Abdellatif 2011).

1.7.4.2 miRNAs in diseases

miRNA functions are implicated in crucial biological processes and alteration of miRNAs expression has been linked to pathogenesis of several human diseases including cancer, cardiovascular, immune, and neurological disorders (Tufekci et al. 2014). For instance, oncogenic miRNAs (oncomirs), miR-9, miR-17-92, miR-21, miR-27a, miR-103, miR-106, miR-107, miR-125b, miR-155, miR-221/222 are up-regulated in many cancers (Tufekci et al. 2014, Hayes et al. 2014). In immune diseases, miR-146, miR-155, miR-203 and miR-346 were found up-regulated in rheumatoid arthritis (Furer et al. 2010, Stanczyk et al. 2011). Up-regulation of miR-9, miR-125, and miR-128 has been associated with Alzheimer disease (De Smaele et al. 2010). Moreover, deregulated expression of miR-1, miR-208, miR-215, miR-487a and miR-502 has been implicated in heart diseases (Wang et al. 2014, Duan et al. 2014). Several miRNAs including let-7, miR-9, miR-15, miR-16, miR-34 and miR-125 were also found down-regulated in endometriosis (Santamaria und Taylor 2014)

1.7.4.3 miRNAs and pregnancy

Nowadays, it is well documented that human placenta and trophoblast cells express a specific profile of miRNAs in which three miRNA clusters are predominately expressed: the chromosome 19 miRNA cluster (C19MC), the chromosome 14 cluster (C14MC) and the miRNA 371-3 cluster which included in the reviews (Bentwich et al. 2005, Barad et al. 2004, Morales-Prieto et al. 2013). C19MC is a placental-specific miRNAs cluster expressed only in primates, while C14MC, also referred as miR-379/miR-659 cluster is expressed in all placental mammals (Flor und Bullerdiek 2012, Noguer-Dance et al. 2010, Morales-Prieto et al. 2013, Glazov et al. 2008). miRNA 371-3 cluster is also conserved among mammals (Laurent et al. 2008). However, not all miRNAs in these clusters are conserved between species and thus, an intensive study of species specific miRNA in placenta has been performed in order to link genomic diversity and the miRNA clusters in evolution (include in this thesis) (Morales-Prieto et al. 2014). In humans expression of C19MC and C14MC changes throughout pregnancy and it is contained to trophoblast cells. Comparison of miRNAs expression between first trimester and third trimester placenta showed that 5 out of 10 miRNAs that highly expressed in third trimester placenta tissue were C19MC miRNAs (miR-516-5p, miR-520d, miR-524, miR-524*, miR-525*), but none of them were detected in first trimester placenta. This finding suggested that these miRNAs are possibly connected to placental development (Luo et al. 2009). In addition, Donker *et al* showed that C19MC miRNAs were highly expressed in primary third trimester trophoblast cells (Donker et al. 2012). Consistently, the results of our group in primary trophoblast cells have demonstrated high expression of C14MC miRNAs in first trimester which decreased towards third trimester, while C19MC miRNAs were low expressed at the beginning of pregnancy but highly expressed in third trimester trophoblast cells (Morales-Prieto et al. 2013, Morales-Prieto et al. 2012).

Furthermore, Wang D. *et al* demonstrated that two C19MC miRNAs (miR-517b and miR-519b) are mainly located in trophoblast layer of first trimester placenta, which indicated that these miRNAs were probably involved in regulation of trophoblast proliferation during first trimester pregnancy (Wang et al. 2012a). Recently, more

evidence has been accumulated demonstrating that C19MC miRNAs contribute to migration of trophoblast cells more than to their invasive properties (Xie et al. 2014).

Table 1. miRNAs related trophoblast cell functions

miRNAs	Chromo-some	Regulatory trophoblast functions	Target genes	Cell models	References
miR-16	13	Proliferation ↓ Invasion ↓ Angiogenesis ↓	CCNE1	dMSC HTR-8/SVneo HUVEC	(Wang et al. 2012c)
miR-19b	13	Differentiation ↓ Proliferation ↑	hCYP191A1, hCGß, GCM1	Isolated trophoblast cells	(Kumar et al. 2013)
miR-20b	X	Angiogenesis ↑ Differentiation ↑	ephrin-B 2 EFNB4	HUVEC BeWo Placental tissues	(Wang et al. 2012b)
miR-21	17	Proliferation ↑ Invasion ↑	PTEN	TCL-1	(Maccani et al. 2011)
miR-21	17	Vascular resistance ↑	CSE	Placenta tissue	(Cindrova-Davies et al. 2013)
miR-29b	7	Invasion ↓ Angiogenesis ↓ Apoptosis ↑	MCL1, MMP2, VEGFA, ITGB1	HTR-8/SVneo BeWo	(Li et al. 2013)
miR-34a	1	Proliferation ↓ Invasion ↓	NOTCH1, JAG1	JAR	(Nadeem et al. 2011)
miR-106a	X	Differentiation ↓ Proliferation ↑	hCYP191A1, hCGB, GCM1	Isolated trophoblast cells	(Kumar et al. 2013)
miR-141	12	Proliferation ↓	?	JEG-3	(Morales-Prieto et al. 2011)
miR-144	17	Proliferation ↑ Invasion ↑	Titin	Isolated trophoblast cells	(Liang et al. 2014)
miR-155	21	Proliferation ↓ Migration ↓	CCND1	HTR-8/SVneo	(Dai et al. 2012)
miR-155*	21	AP-1/NF-κB ↓ pathway	PTEN	HTR-8/SVneo	(Xue et al. 2013)
miR-195	17	Invasion ↑	ActRIIA	HTR-8/SVneo	(Bai et al. 2012)
miR-210	11	Migration ↓ invasion ↓	*EFNA3*, *HOXA9*	CTBs (1st trimester)	(Zhang et al. 2012)
miR-210	11	invasion ↓	ISCU	Swan 71	(Lee et al. 2011)
miR-210	11	?	HSD17B1	BeWo	(Ishibashi et al. 2012)
miR-218	4	Proliferation ↓	FBXW8	JEG-3	(Shi et al. 2014)

miR-367c	4	Proliferation ↑ Migration ↑ Invasion ↑	ALK5 ALK7	HTR-8/SVneo Placental explant	(Fu et al. 2013b)
miR-378a-5p	5	Proliferation ↑ Invasion ↑ Migration ↑	NODAL	HTR-8/Svneo Placental explant	(Luo et al. 2012)
miR-378a-5p	5	Fusion ↓ Differentiation ↓	CCNG2	BeWo	(Nadeem et al. 2014)
miR-424	X	Differentiation ↑	FGFR1	Isolated trophoblast cells	(Mouillet et al. 2013)
miR-512-3p	19	ß-galactosidase activity ↓	PPP3R1	BeWo	(Kurashina et al. 2014)
miR-517b, miR-519a	19	Proliferation ↓	?	Placenta villi	(Wang et al. 2012a)
miR-518c	19	?	HSD17B1	BeWo	(Ishibashi et al. 2012)
miR-519d	19	Migration ↓	CXCL6, NR4A2, FOXL2	HTR-8/SVneo	(Xie et al. 2014)
miR-675	11	Proliferation ↓	NOMO-1	JEG-3	(Gao et al. 2012)

↓ Decreased; ↑ increased ; ? not known

hCYP191A1, human aromatase; hCGß, human chorionic gonadotropin; GCM1, human gial cell missing; CSE, cystathionine β-synthase and cystathionine γ-lyase; EFNA3, Ephrin-A3; HOXA9, Homeobox protein Hox-A9; ISCU, Iron-sulfur cluster scaffold; HSD17B1, Hydroxysteroid (17-beta) dehydrogenase 1; ALK5, Activin receptor-like kinase 5; ALK7, Activinreceptor-like kinase 7; ActRIIA, Activin Receptor IIA; NOMO-1, NODAL modulator 1; PTEN, Phosphatase and tensin homolog; CCND1, Cyclin D1; CCNE1, Cyclin E1; CCNG2, Cyclin G2; JAG1, JAGGED 1; MCL1, Myeloid cell leukemia sequence 1; MMP-2, Matrix metalloproteinase-2; VEGFA, Vascular endothelial growth factor A; ITGB1, integrin beta 1; PPP3R1, protein phosphatase 3, regulatory subunit B, alpha; CXCL6, Chemokine (C-X-C motif) ligand 6; nuclear receptor subfamily 4, group A, member 2, NR4A2; FOXL2, forkhead box L2.

miRNAs exhibit several functions during pregnancy by binding to their target genes which are responsible for trophoblast cell functions including cell proliferation, migration, invasion, apoptosis and angiogenesis (Table 1). Differential expression patterns of placenta miRNAs during different stages of placental development suggest that miRNAs regulate placental development and have a gestational stage specific function (Fu et al. 2013a). Altered miRNAs expressions have been associated with pregnancy complications including preeclampsia (PE), intrauterine growth restriction (IUGR), preterm birth, small for age (SGA) and Gestational Diabetes Mellitus (GDM) (Morales-Prieto et al. 2014, Fu et al. 2013a, Zhao et al. 2013).

Currently, miRNAs have been considered are novel potential biomarkers for diagnosis, prognosis and therapeutic targets in various diseases including pregnancy complications (Enfield et al. 2012, Anton et al. 2013, Santamaria und Taylor 2014, Traver et al. 2014, Morales-Prieto et al. 2014). It has been reported that placenta-specific miRNAs are secreted into maternal circulation by trophoblast cells via exosomes (Luo et al. 2009, Donker et al. 2012). Therefore, expression of these miRNAs in maternal circulation could be useful as possible marker of prognosis for pregnancy associated diseases (Miura et al. 2010, Kotlabova et al. 2011).

The work of our group revealed that miR-21 is the highest expressed miRNA in primary first trimester trophoblast cell (out of 762 miRNAs). Moreover, miR-21 is also highly expressed in HTR-8/SVneo, ACH-3P, AC1-M59, JEG-3 and primary third trimester trophoblast cells (Morales-Prieto et al. 2012). *MIR*-21 gene is located in chromosome 17 and its deregulation has been associated with cancer, cardiovascular diseases and inflammation (Li et al. 2012). Remarkably, miR-21 is low expressed in the placenta of newborns suffering from fetal growth restriction (Maccani et al. 2011) and also down-regulated in severe preeclampsia placenta (Choi et al. 2013). Thus, it is expect that miR-21 may has an important regulatory function during pregnancy.

Chapter 2 Objectives

2.1. General Objective

The general objective of this study was to elucidate the intracellular pathways responsible for the signaling of LIF, OSM and miR-21 and to investigate their role in controlling trophoblast cell functions.

2.2. Specific Objectives

- To identify the effects of cytokines expressed during implantation on trophoblast cell functions in patho- and physiological conditions.
- To compare the function of LIF and OSM on the regulation of trophoblast cell proliferation and invasion through activation of JAK/STAT pathway.
- To summarize the current knowledge on placental miRNAs, their origin, expression in human placenta with special regard to trophoblast cells, and their potential future as biomarkers.
- To compare the miRNA profile of primary first and third trimester trophoblast cells with that of different trophoblastic cell lines.
- To elucidate the role of miR-21 in the regulation of trophoblast cell functions (proliferation, migration and invasion) and its influence on involved intracellular signal cascades.

Chapter 3 Publications overview

Six publications are included in this dissertation: Three review and three original research articles. The study plan of these papers is described in Figure 10. Briefly, trophoblastic cell lines were stimulated with LIF or OSM, and subsequently, the activation of STAT3 and ERK1/2 was analyzed. Furthermore, effects of LIF and OSM treatments were investigated on cell proliferation and invasion. The second main line of this thesis investigated the expression of miRNAs in different cellular models and compared it with primary trophoblast cells. Finally, miR-21 expression was overexpressed and knocked down in two trophoblastic cell lines and the function and potential targets of miR-21 were investigated.

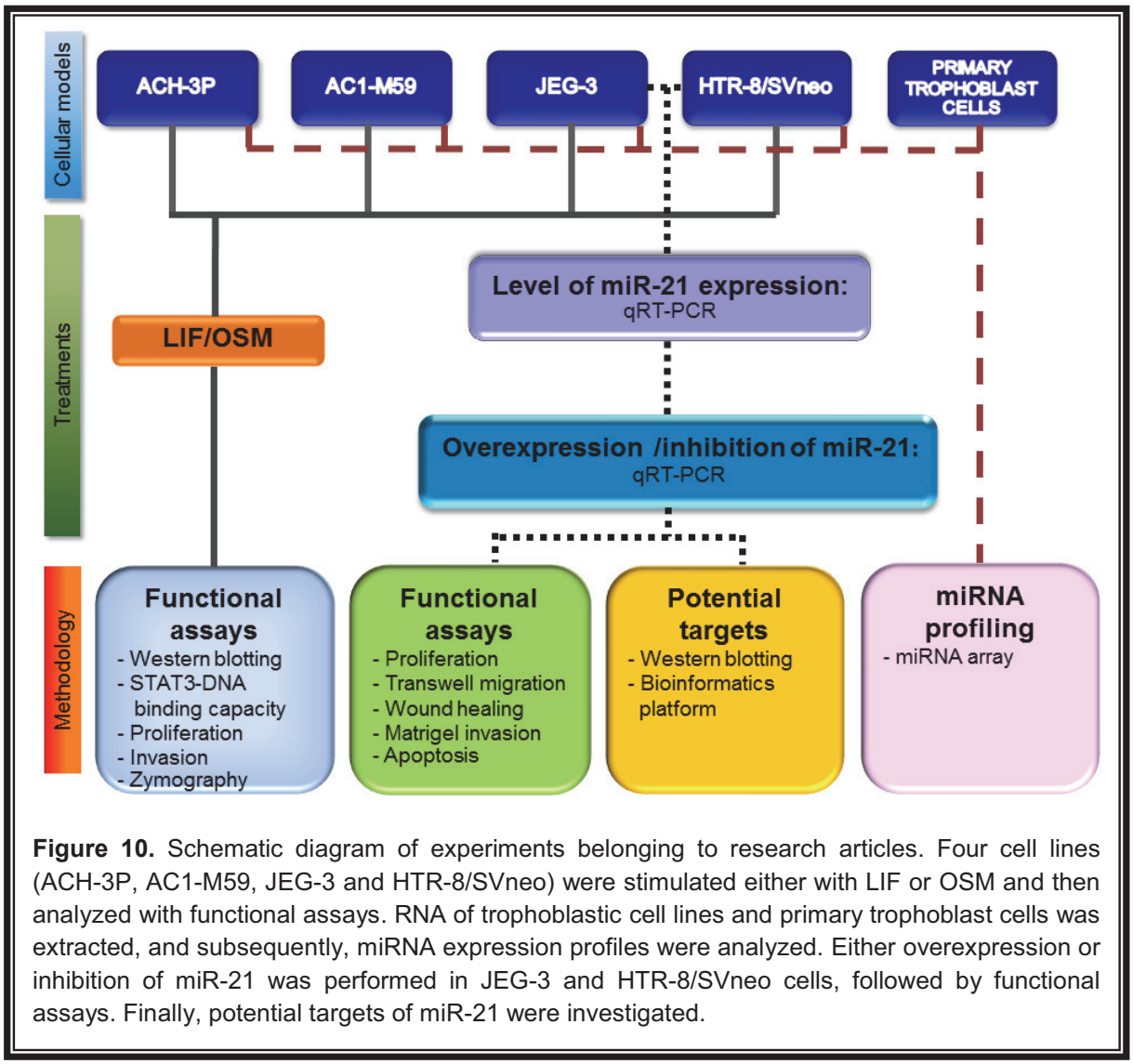

Figure 10. Schematic diagram of experiments belonging to research articles. Four cell lines (ACH-3P, AC1-M59, JEG-3 and HTR-8/SVneo) were stimulated either with LIF or OSM and then analyzed with functional assays. RNA of trophoblastic cell lines and primary trophoblast cells was extracted, and subsequently, miRNA expression profiles were analyzed. Either overexpression or inhibition of miR-21 was performed in JEG-3 and HTR-8/SVneo cells, followed by functional assays. Finally, potential targets of miR-21 were investigated.

Chapter 4 Results

4.1 Part I: Cytokines regulate trophoblast cell behavior

Publication lists

4.1.1 **Cytokines regulating trophoblast invasion**

Fitzgerald JS, Abad C, Alvarez AM, Bhai Mehta R, **Chaiwangyen W**, Dubinsky V, Gueuvoghlanian B, Gutierrez G, Hofmann S, Hölters S, Joukadar J, Junovich G, Kuhn C, Morales-Prieto DM, Nevers T, Ospina-Prieto S, Pastuschek J, Pereira de Sousa FL, San Martin S, Suman P, Weber M, Markert UR. *Advances in Neuroimmune biology*. 2011; 2 (1): 61-97

This review paper demonstrates that cytokines play an important role in different processes and regulate trophoblast cell functions during human pregnancy.

4.1.2 **Oncostatin M and leukemia inhibitory factor trigger STAT3 and ERK1/2 pathways but result in heterogeneous cellular responses in trophoblastic cells**

Chaiwangyen W, Ospina-Prieto S, Morales-Prieto DM, Pereira de Sousa FL, Pastuscheck J, Fiztgerald JS, Schleussner E, Markert UR.. *Reprod Fertil Dev*. 2014 Sep 24

LIF and OSM are IL-6 cytokine family and this study show that LIF shares with OSM the capacity of STAT3 and ERK1/2 phosphorylation in all trophoblastic cell lines whereas their biological effects are cell type dependent.

Advances in Neuroimmune Biology 2 (2011) 61–97
DOI 10.3233/NIB-2011-023
IOS Press

Cytokines Regulating Trophoblast Invasion

Justine S. Fitzgerald[a,*], Cilia Abad[b], Angela M. Alvarez[c], Ratnesh Bhai Mehta[d], Wittaya Chaiwangyen[a], Valeria Dubinsky[e], Barbara Gueuvoghlanian Silva[f], Gabriela Gutierrez[e], Simone Hofmann[g], Sebastian Hölters[a], Jennifer Joukadar[h], Gisela Junovich[e], Christina Kuhn[g], Diana M. Morales-Prieto[a], Tania Nevers[h], Stephanie Ospina-Prieto[a], Jana Pastuschek[a], F. Lazaro Pereira De Sousa[a], Sebastian San Martin[i,j], Pankaj Suman[k], Maja Weber[a] and Udo R. Markert[a]

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Abstract. Pregnancy is personally special to every woman expecting a child, but is also interesting from the perspective of an immunologist. During a physiological pregnancy, the mother's immune system decides to tolerate and foster an incorporated, non-self, non-dangerous organism. Whether the maternal reaction stems from deciphering the foreigness or safeness of this new individual, it is the general consensus that there is a foeto-maternal, bidirectional "dialogue" occurring and that the "messages" that are "spoken" are relayed through signaling mediators, which are capable of transmitting a functional command to a target cell. Much information dedicated to this theme has been gleaned in the past decade; however, the complex nature of cytokine networks jeopardizes clarity.

In this review, we touch upon a list of mediators that are vital for reproduction. These factors are divided according to their receptor family, because this elucidates the characteristic signal transducing pathway, which is expected to mediate their signal within the target cell. The target cells of interest are the trophoblast, upon which we focus for several reasons: 1. the trophoblast represent the foetal compartment while participating in foeto-maternal interplay (e.g. while invading the decidua, trophoblasts are in constant communication with uterine, maternal immunocytes, which check and contain this function), 2. trophoblasts are responsible for foetal well-being (e.g. nutrition, protection from the environment) and 3. dysfunctional trophoblast function results in several pregnancy complications (e.g. preeclampsia, intrauterine growth retardation, miscarriage, preterm delivery).

We summarize what is described in the literature on how these mediators are distributed within the reproductive tract, which cells are expressing their respective receptors (especially which trophoblast subsets) and how they modify trophoblast function

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(namely invasion, proliferation, differentiation and apoptosis). Furthermore, we unearth for which mediator the signal transducing pathway is verifiably used in trophoblast (ic) cells. Finally, we correlate actual biological importance of the mediator for reproduction by comparing murine knockout phenotypes and known positive and negative associations of these mediators with human pregnancy pathologies (as listed above). We expect this concise review to be useful to both basic researchers and clinicians who wish to obtain an overview of the reproductive cytokine network in respect to the trophoblast.

Keywords: Placenta, pregnancy, trophoblast, cytokines, cytokine receptors, chemokine receptors, immunoregulation

INTRODUCTION

The immunological situation found during pregnancy is of special importance. Most often, pregnancy, although generally accepted as a miraculous milestone in the lifeline of a woman, is not perceived as a critical condition in which the mother is incorporating a complex organism of foreign origin. Indeed, normal and physiological pregnancies are usually concluded in an uncomplicated fashion without the maternal organism reacting in any adverse way toward the foetus or placenta inside the gravid uterus, so that the accomplishments of this non-reaction are quite ignored [1].

During pregnancy, it is of utmost consequence that the so-called foreign object, the foetus, is not recognized as such, but instead accepted as a "friend". There are two most prominent theories committed to explaining this physiology. One maintains that an active *induction* of tolerance of the foetal allograft is initiated through *bidirectional* dialogue between the foetus (or placenta) and the mother during physiological pregnancies [2]. Another, newer hypothesis, which proposes that the immune system is more concerned with damage than with foreignness, describes that without a so-called "danger signal" stemming either directly from the pregnancy or from a precarious setting during pregnancy, the foetus will not be recognized as anything that requires an aggressive immunological response [3, 4]. The discussion between both of these fields is quite controversial and does not promise to be resolved completely in the near future (reviewed in [5, 6]). However, both sides realize that a major contributor to any immunological reaction that might be seen during pregnancy would be identified, amongst others, per cytokines [5, 7].

Cytokines, being the main mode of communication between immunological cells and their targets, would be instigators of tolerance, rejection or any other immunological reaction toward a pregnancy. When these signals are intercepted, blocked or amplified, dire consequences can be expected. During pregnancy, communication between the foetus (placenta) and the mother (decidua) is an intricate network intercalated with that of the hormonal network. Cytokines are produced by immunocytes that are in dialogue with their environment, and these immunocytes are in turn responsive to other cytokines. Many of these cytokines are produced in a spaciotemporal fashion, indicating that they are in cinque with maternal pregnancy homeostasis, and are responsible for the fine tuning of specific functions within the placenta.

In this review, we focus on a spectrum of cytokines which are known to be important for reproduction. We are mainly interested in their effects on the trophoblasts, a main subset of cells that constitute the placenta and which are of foetal origin. Maternal, uterine immunocytes come into direct contact with trophoblast cells, thus initiating a bi-directional transfer of information.

In short, there are three main trophoblast subsets in the placenta: the villous cytotrophoblast (CTB), the syncytiotrophoblast (STB) and an intermediate trophoblast subset that is also termed extravillous trophoblast (EVT). The first two subsets are found coating the villous tree of the placenta. The STB layer is found on the outside coating, and comes into direct contact with maternal blood of the intervillous space (and with maternal immunocompetent cells), and thus is also responsible for such jobs as transportation of nutrition and oxygen to the foetus. It is also important in metabolic changes including detoxification and protection from microbes. The layer just underneath the STB consists of CTB, which are often considered a sort of trophoblast stem cell that replenishes the STB layer when areas of the villous tree thin out and are exhausted [8, 9]. STB consist namely of fused CTBs, which after fusion, have become aproliferative, meaning that the only manner in which the STB layer can grow, is through a constant replenishment from the below CTB layer [9]. The CTB however have several functions: they either differentiate to replenish STB or they differentiate along the invasive pathway. This situation occurs in areas where the floating villi are attached to the basement membrane. Upon doing so, some CTB differentiate first to anchor the villi to the basement membrane, but some further differentiate into an

survival of myeloid leukocytes and their precursors [43]. The receptor of GM-CSF consists of α (GM-Rα) and β subunits. The β subunit is shared with receptors for GM-CSF, IL-3 and IL-5 [44]. On the cell membrane of choriocarcinoma cell lines such as BeWo, JEG-3 and JAR and of primary trophoblast cells (CTB and EVT; only weak expression on STB), GM-Rα protein can be detected [45]. Researches demonstrate, also, a role in the modulation of Th1 and Th2 immune responses for this cytokine [46, 47]. Studies of *in vitro* angiogenesis assays and *in vivo* Matrigel plug assays with endothelial cell of mice indicated that GM-CSF and monocytes play a key role in angiogenesis through the regulation of vascular endothelial growth factor (VEGF) [48]. Acting as an immunoregulatory agent, GM-CSF, which is regarded as an important determinant of pregnancy outcome, contributes to regulation of placental morphogenesis and maternal immune tolerance [49]. Furthermore, as an embryotrophic factor, it is indispensable for providing ideal foetal evolution after implantation, such as foetal and post-natal growth, and the likelihood of obesity in adult descendants and it regulates the morphological and functional development of the placenta [50]. It contributes as a driving force in a tightly regulated sequence of events involving CTB cell proliferation and terminal differentiation to generate STB cells [51]. By accommodating these trophoblastic functions, it is believed to be involved in invasion of maternal decidual tissues and blood vessels, although actual *in vitro* data is yet to be generated.

GM-CSF seems relevant to human reproductive medicine, since its deficiency is associated with placental insufficiency, as well as immunological disorders, and it is shown to be involved in miscarriage, low birth weight, pre-term delivery and preeclampsia [49]. According to other studies concerning these complications, GM-CSF expression is increased in the decidua of preeclamptic women and mice [52]. During the post-conceptional period, the GM-CSF which is secreted into the uterus and the salpinges is implicated as a regulator of the growth and development of the pre-implantation embryo [53]. One study of women suffering from recurrent miscarriage showed that an increase in serum GM-CSF content, which is seen in normal pregnancy, did not occur in the miscarriage group [54]. Researchers have demonstrated that the outcome of gestation is radically modified by the administration of exogenous GM–CSF to mice [55]. Other studies in mice with a null mutation in the GM-CSF gene show that fertility and the number of surviving pups are impaired in the absence of GM-CSF [56–58]. These studies reveal that a GM-CSF deficiency leads to growth retardation and small litter sizes probably secondary to placental anomalies, including a diminished proportion of glycogen cells in the spongiotrophoblast layer [56].

Uterine and placental tissues are recognised as potent sources of hematopoietic colony stimulating activity [59, 60]. GM-CSF is produced by uterine epithelial cells, and GM-CSF is found in the luminal and glandular epithelium. GM-CSF synthesis by uterine epithelial cells is predominantly stimulated by estrogen; its expression stays high for the first few days after conception, but then declines around the time of embryo implantation, which occurs under the inhibitory influence of progesterone [61]. Once implantation begins, cell lineages in the chorionic villi of the early developing placenta contribute to GM-CSF production including the invading CTB cells [62], villous fibroblasts [63], and placental macrophages [64]. Other analyses also demonstrated that in women and in mice, GM-CSF synthesis by reproductive tract epithelial cells is responsive to ovarian steroid hormones and to male seminal fluid factors (59, 60).

Several identified polymorphisms in the genes that encode GM-CSF are identified conferring endogenous variability in GM-CSF bioavailability and signaling networks [65–67]. At least three signaling pathways have been described for this cytokine: the mitogen-activated protein kinase (MAPK) pathways, the JAK/STAT pathway and the phosphatidylinositol 3-kinase pathway (PI3K) [68–72]. Furthermore, there is one study that supports the idea that STAT5 is recruited to the membrane from the cytosol upon GM-CSF stimulation and is tyrosine-phosphorylated by JAK2 [44], but it is yet to be discovered which pathway is used for signal transduction of GM-CSF in the trophoblast. Our own unpublished results indicate that at least STAT3 is not involved in mediating its signal. At least in the inner cell mass it has been demonstrated that GM-CSF signaling occurs independent of its ß common subunit [53]. Considering the relevance of GM-CSF in the early stages of pregnancy, a complete understanding of its role represents an opportunity for developing interventions for achieving favorable obstetrical outcomes.

Leukemia Inhibitory Factor (LIF)

LIF, a member of the IL-6 family, is a widely known pleiotropic cytokine which posseses a pivotal role in human reproduction [73, 74]. LIF was first identified in 1987 by Metcalf and colleagues as a factor that induced

the differentiation of mouse myeloid leukemic M1 cells into macrophages [75]. Currently, LIF is known to be expressed in numerous cell types including neurons, hepatocytes, and kidney and breast epithelial cells [76, 77] and its main role is the mediation of inflammatory cell responses [78, 79]. Nevertheless, LIF is also recognized to control uterine receptivity for blastocyst implantation, or to influence trophoblast behavior by promoting proliferation, invasion and differentiation [80, 81].

LIF appears to be an important modulator of pregnancy in humans. Both granulosa-lutein cells and ovarian stromal cells expressed LIF mRNA and protein. Furthermore, LIF concentration in follicular fluids correlates with the embryo quality suggesting an important role of LIF in the physiology of ovulation and early embryo development [82]. On the other hand, LIF is expressed by the endometrium, predominantly in the glandular and luminal epithelium, and its concentrations reach maximal levels during the secretory/postovulatory phase of the menstrual cycle, when the implantation is expected to commence [80, 83]. During the implantation window, trophoblast cells also express mRNA for the LIF receptor which maximizes interaction with the endometrium. After adhesion, the blastocyst is able to produce LIF mRNA by itself, which leads to an increase in cell proliferation and triggers differentiation into CTBs and STBs, and enhances invasive behavior of trophoblast cells. [81, 84–86]. The LIF receptor is expressed by both villous as well as EVT cells throughout pregnancy. EVT express the LIF receptor as they pass decidual leukocytes which secrete LIF, and thus they come into dialogue [87].

LIF's crucial role during embryo implantation is evident in LIF deficient female mice, which albeit being infertile by the inability of the blastocyst to attach, could recover fertility by LIF infusion into the uterus [88]. Conversely, LIF receptor knockout mice implant, but exhibit impaired placenta function, which results in death within 24 h of birth [89]. In humans, LIF expression levels are diminished in endometrial cell cultures from infertile women with repeated abortions or unexplained infertility [90, 91]. In fact, women wearing a copper T380A intrauterine device (IUD), one of the most effective anticonceptive devices, showed also lower expression of LIF compare with control [73]. But it is not only LIF protein expression deregulation which may have a negative impact on the pregnancy outcome, functionally relevant mutation of the LIF gene are found higher in infertile women in comparison with fertile controls resulting in poor outcome in IVF treatment [92].

LIF triggers its effects by induction of a signaling heterodimer consisting of the specific LIF receptor (LIFR) and the subunit gp130 [74]. This causes the activation of the RAS/MAPK (RAt Sarcoma/ MAPK) and JAK/STAT cascades [93–95]. STATs are a family of transcription factors located in the cytoplasm, which after activation can form hetero- or homo-dimers and be translocated into the nucleus to control gene expression [96, 97]. STATs are associated with regulation of implantation and maternal immune response in early pregnancy [98]. Furthermore, we have demonstrated that STAT3, a member of the STAT family, plays a crucial role in the regulation of trophoblast invasion mediated by LIF. LIF induces alteration of proteases such as tissue inhibitor of metalloproteinase 1 (TIMP1) and Caspase4 via STAT3, which elevates trophoblast(ic) proliferation and invasion, and STAT3 knockdown annuls these functions even in the presence of LIF [81, 86, 99]. LIF has been patented as a supplement to culture media to promote the development of mammalian embryos to the implantation stage, since growth in the presence of LIF increases the percentage of embryos that reach the implantation stage than growth without LIF (United States Patent 5962321; Inventors: Gough, Nicholas Martin; Willson, Tracey Ann, Seamark, Robert Frederick (Beulah Park, AU), http://www.freepatentsonline.com/5962321.html).

Granulocyte-Colony Stimulating Factor (G-CSF)

G-CSF is a macrophage- and granulocyte-inducing (MGI) protein, mainly produced by macrophages, which induces the proliferation and differentiation of macrophage and granulocyte precursor cells. Furthermore, G-CSF is able to induce terminal differentiation in murine leukemic cells and thereby suppress leukemic cell populations. The murine and human G-CSF protein show almost complete cross-reactivity regarding biological effects and receptor-binding in human or murine normal and leukaemic cells [93–97]. The molecular weight of G-CSF amounts 19.6 kDa, consist of 174 amino acids and is o-glycosylated at Thr-133 [100–102]. The encoding gene of G-CSF is located at chromosome 17, 17q11.2–21 [103]. The G-CSF receptor is a 150 kD single subunit protein [104].

G-CSF is produced by those decidual cells that are in contact with anchoring villi but not by trophoblast cells of the chorionic villi [105]. G-CSF-receptor (G-CSFR) is expressed in human placental membranes as well as CTBs and STBs and decidual stromal and endometrial gland cells [104, 105]. G-CSFR is intensely expressed in first and third trimester, but not

containing VEGF showed that *in vitro* migration of JEG-3 cells through a transwell membrane was significantly reduced by silencing of VEGF. Therefore, a role of VEGF was postulated in the regulation of trophoblast migration [25]. It is also known that VEGF acts via autocrine stimulation loop in trophoblastic cells [409]: in normal human CTB, TGF-beta1 induced an hypoxia inducible factor-1α (HIF-1α)-mediated VEGF secretion (and a TGF-ß1-stimulated-ERK1/2 activation may be involved in this process) [410]. Moreover, both the PI3K–AKT–mTOR (mammalian Target of Rapamycin)–HIF-1α and ERK–HIF-1α signaling pathways are crucial for increasing VEGF and endoglin expression in response to hypoxia in BeWo cells [411].

It has been shown that VEGF is predominantly expressed in tumour cell lines derived from female reproductive organs [412]. In tumors, VEGF is known to significantly contribute to pathological angiogenesis, tortuosity of tumor vasculatures and vasculogenesis, which all together lead to accelerated growth rates of tumors, invasion and metastasis [413]. VEGF family members regulate CTB survival and expression of a subset of its family members is dysregulated in severe forms of preeclampsia [414]. Currently, assays have been implemented clinically to detect the concentration of soluble Flt-1 (sFlt-1), which binds and inactivates VEGF, and PlGF in maternal serum at mid-gestation. Unfavorable ratios of sFlt-1/PlGF can predict the development of PE [415].

Considering all these data, VEGF is most likely involved in many processes related to reproductive physiology, and which are essential for correct implantation and placentation.

Hepatocyte Growth Factor (HGF)

HGF is a pleiotropic cytokine whiuch was first defined as a potent mitogen for hepatocytes *in vitro* [416, 417]. HGF acts through binding to the receptor, c-Met, causing tyrosine kinase activation and autophosphorylation at tyrosine residues [418]. Following auto- phosphorylation, the PI3K and MAPK pathways are two main signaling cascades involved in mediating the HGF signal [419]. HGF is associated with cell proliferation, differentiation, invasion and angiogenesis in other cell systems [420].

The placenta has been shown to express a significant amount of HGF [416]. Serum of HGF levels increase throughout the first, second and third trimesters of pregnancy, respectively [417]. Furthermore, the second trimester period displays the highest HGF level in amniotic fluid, but the rate of HGF production from placental tissue is not significantly different between the three periods [417]. Mesenchymal cells of the villi express HGF mRNA in situ and CTB express HGF receptor, c-Met [421, 422]. In addition, c-Met protein is expressed in choriocarcinoma cell lines [423, 424].

In vivo "gene knockout" studies in mice lacking the HGF gene show placental defects and embryonic lethality which were identified as a complete lack of development of labyrinthine trophoblast at 13.5–14.5 ([425] and reviewed in [426]). A single injection of HGF/SF at embryonic day 9.5 (E9.5) into the amniotic cavity of HGF/SF −/− embryos rescued the placental defect [427]. In c-Met mutant mouse embryos, identical placental and additional liver abnormalities were found [428]. Transgenic mice that overexpress HGF, seem to have lower tumor yields or a lower incidence of hepatocellular cancers [429].

HGF stimulates nitric oxide (NO) synthesis involved in the human EVT cell line SGHPL-4 invasion of fibrin gel [430]. HGF regulates trophoblast invasion through the activation of c-Met and consequent secretion of 92-kDa collagenase as determined by the Boyden chamber invasion assay [431]. Trophoblast motility is an important step for the invasive process. Cartwright et al found that HGF stimulates SGHPL-4 motility and invasion by activation of the PI3K pathway [432]. Inhibition of the MAPK pathway also inhibited HGF-induced motility of primary human EVT, whereas not effect on basal motility [432]. The homeobox gene HLX is expressed in proliferating and migrating (but not invading) human trophoblast cells and HLX expression is significantly decreased in human IUGR. In SGHPL-4 and HTR-8/SVneo cells, HGF stimulated the production of HLX mRNA and protein expression, which resulted in increased trophoblast cell migration. Reciprocally, HLX inactivation significantly decreased trophoblast migration, thus the HLX gene is also a key of trophoblast cell migration via HGF/c-Met signaling pathway [238]. Finally, although much attention has been dedicated to HGF and its effects on trophoblast invasion, only little to no attention is paid to its effects on trophoblast proliferation.

As to be expected from the above studies, HGF seems to be associated with IUGR. HGF and c-met expression are reduced in IUGR placentae [433]. Recently, plasmatic HGF measurements at around mid-gestation (14–20 gestational weeks) were successfully implemented as a predictive marker for small-for-gestational age foetuses, but not for preeclampsia [434]. In this context, no direct information can

be gleaned thus far from the literature on an association between preeclampsia and HGF although HGF is reported to have an effect on trophoblast invasion. In this aspect, it is interesting that the hypothesis of trophoblast invasion being causative for preeclampsia has been challenged [435]. It has proposed instead that trophoblast invasion is causative of IUGR and related alterations of foetal growth, which is in line with the information available thus far on HGF and its association to foetal growth retardation. Also in line with the above findings, HGF seems to be associated with trophoblastic disease [436]. Miscarriage, spontaneous abortion and preterm delivery have also not been associated with HGF yet.

CONCLUSION

It may be concluded that cytokines play a vital role in mammalian reproduction, including during human pregnancy. Much information has been unearthed in terms of where and when these cytokines are produced, and which cells possess receptors, indicating that specific cells within the reproductive tract are potentially capable of reacting to its corresponding cytokine. In the past decade, a tremendous array of data has been generated concerning the functional actions of these cytokines on trophoblast populations. This denotes the vast interest and potential that many international scientists believe this field of research occupies. This is in part due to the impact that is supposed to emanate from the results found in these studies in terms of preeclampsia and IUGR, but also of cancer.

It must be stressed however, that we are not close yet from reaching a therapeutic goal. This review exposes several research gaps, especially in terms of factual knowledge on signal transduction in the trophoblast. It may not be assumed that the mode of signal transduction for a specific mediator in a specific cell will be the same in the trophoblast, or even between the trophoblast(ic) subsets. It is vital to comprehend the exact regulating mechanisms of signaling mediators if these mediators are proposed for therapeutic interventions. In the example of LIF, for instance, the negative feedback mechanism driven through the STAT signaling system warrants caution in using LIF as a therapeutic agent, since both low as well as high LIF concentrations would results in an under-utilization of STAT3. This would probably negatively regulate trophoblast invasion. Therefore, understanding trophoblast(ic) signaling functions well should help to enforce innovation towards novel therapeutic approaches that will assist in enhancing reproduction on the one side, and on the other, combating cancer.

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Oncostatin M and leukaemia inhibitory factor trigger signal transducer and activator of transcription 3 and extracellular signal-regulated kinase 1/2 pathways but result in heterogeneous cellular responses in trophoblast cells

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Abstract. Leukaemia inhibitory factor (LIF) and oncostatin M (OSM) are pleiotropic cytokines present at the implantation site that are important for the normal development of human pregnancy. These cytokines share the cell membrane receptor subunit gp130, resulting in similar functions. The aim of this study was to compare the response to LIF and OSM in several trophoblast models with particular regard to intracellular mechanisms and invasion. Four trophoblast cell lines with different characteristics were used: HTR-8/SVneo, JEG-3, ACH-3P and AC1-M59 cells. Cells were incubated with LIF, OSM (both at 10 ng mL^{-1}) and the signal transducer and activator of transcription (STAT) 3 inhibitor S3I-201 (200 μM). Expression and phosphorylation of STAT3 (tyr^{705}) and extracellular regulated kinase (ERK) 1/2 ($thr^{202/204}$) and the STAT3 DNA-binding capacity were analysed by Western blotting and DNA-binding assays, respectively. Cell viability and invasiveness were assessed by the methylthiazole tetrazolium salt (MTS) and Matrigel assays. Enzymatic activity of matrix metalloproteinase (MMP)-2 and MMP-9 was investigated by zymography. OSM and LIF triggered phosphorylation of STAT3 and ERK1/2, followed by a significant increase in STAT3 DNA-binding activity in all tested cell lines. Stimulation with LIF but not OSM significantly enhanced invasion of ACH-3P and JEG-3 cells, but not HTR-8/SVneo or AC1-M59 cells. Similarly, STAT3 inhibition significantly decreased the invasiveness of only ACH-3P and JEG-3 cells concomitant with decreases in secreted MMP-2 and MMP-9. OSM shares with LIF the capacity to activate ERK1/2 and STAT3 pathways in all cell lines tested, but their resulting effects are dependent on cell type. This suggests that LIF and OSM may partially substitute for each other in case of deficiencies or therapeutic interventions.

Additional keywords: invasion, matrix metalloproteinases.

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Introduction

The interleukin (IL)-6 cytokine family comprises IL-6, IL-11, leukaemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF) and cardiotrophin (CT-1), all of which share the subunit glycoprotein (gp) 130 receptor (Bruce *et al.* 1992; Gearing 1993; Heinrich *et al.* 1998). These cytokines have been studied intensively due to their function as regulators of cellular processes. LIF was first described as inducing differentiation in the murine leukaemia cell line M1 (Gearing *et al.* 1987; Mathieu *et al.* 2012), whereas OSM was identified as a growth regulator in U937 histiocytic lymphoma cells (Zarling *et al.* 1986).

Several reports have demonstrated biological effects of OSM and LIF in a variety of tissues, but these effects seem to be cell type dependent. Although OSM inhibits growth in breast, lung, stomach, ovary and skin tumour cell lines (Horn *et al.* 1990), it may enhance cell proliferation of normal fibroblast, muscle and endothelial cells (Grove *et al.* 1993). Functions of LIF in different cell types include induction of cell proliferation, cell differentiation and cell survival (Hilton 1992).

Both cytokines appear to be important for the normal development of human pregnancy. In fertile women, LIF has been detected in uterine flushings throughout the peri-implantation period and its level increases slightly after the release of LH (Laird *et al.* 1997). In addition, *lif*-deficient mice are infertile due to a lack of blastocyst attachment, but fertility can be recovered by further administration of LIF (Stewart *et al.* 1992). In addition, mutation of LIF receptor (LIFR) disturbs

physiological placentation in mice (Ware *et al.* 1995). In wild-type mice, OSM is transiently expressed in the luminal epithelium on late Day 4 and then in the stroma at the attachment site on Days 5–6 of pregnancy, but not in *lif*-null animals (Fouladi-Nashta *et al.* 2005). Previous studies in humans did not detect OSM transcripts in the endometrium (Cullinan *et al.* 1996).

Levels of OSM are significantly increased in serum from pregnant compared with non-pregnant women (Ogata *et al.* 2000). During the first trimester of pregnancy, OSM is produced by decidual glands and stromal cells and promotes human chorionic gonadotrophin (hCG) release (Ogata *et al.* 2000). Levels of OSM in the placenta and serum of pre-eclamptic women are significantly higher than in normal pregnancy and OSM expression in the cytotrophoblast, syncytiotrophoblast and epithelium of pre-eclamptic placentas is stronger than that of normal placentas (Lee *et al.* 2009).

LIF uses LIFR-gp130, whereas OSM has two binding receptor complexes consisting of OSM receptor (OSMR)-gp130 and LIFR-gp130 (Gearing *et al.* 1992; Thoma *et al.* 1994; Liu *et al.* 1998; Lass *et al.* 2001; García-Tuñón *et al.* 2008). OSM binding to OSMR-gp130 induces Janus kinase (JAK) 2 and signal transducer and activator of transcription (STAT) 3 phosphorylation (Fossey *et al.* 2011). Binding of LIF to its receptor complex activates the JAK/STAT, mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/AKT pathways (Aghajanova 2010). In several cell types, ERK1/2 can be activated by LIF and OSM (Smyth *et al.* 2008; Prakash *et al.* 2011). The ERK/MAPK pathway contains a cascade of protein kinases and regulates a variety of cellular responses, including proliferation, differentiation, survival and apoptosis (Shaul and Seger 2007; Wortzel and Seger 2011).

It has been reported that trophoblast invasion and placental development are regulated by LIF (Bischof *et al.* 1995; Sharkey *et al.* 1995; Nachtigall *et al.* 1996). In particular, our group has shown that LIF enhances the invasiveness of trophoblast cells by activation of STAT3 and downregulation of tissue-specific inhibitors of metalloproteinase (TIMP)-1 (Fitzgerald *et al.* 2005; Poehlmann *et al.* 2005). Extravillous trophoblast (EVT) cells invade the decidua during the first trimester of pregnancy where they are involved in angiogenesis and vascularisation (Lunghi *et al.* 2007; Bilban *et al.* 2010; Fitzgerald *et al.* 2011). EVT invasion is a complex process that requires expression and activation of matrix metalloproteinases (MMPs; Fitzgerald *et al.* 2011; Knöfler and Pollheimer 2012). The MMPs are zinc-dependent endopeptidases capable of degrading components of the extracellular matrix (ECM) that are involved in physiological and pathological events (Staun-Ram and Shalev 2005; Kessenbrock *et al.* 2010). MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are key enzymes for trophoblast invasion during the implantation process (Staun-Ram and Shalev 2005; Cohen *et al.* 2006). Activation of the STAT3 pathway leads to upregulation of MMP-2 expression in tumour cells, as shown in a melanoma cell line (Xie *et al.* 2004).

In the present study we used four different cells lines that are frequently used as models for trophoblast cells but differ in terms of proliferation rates and invasiveness. The cell lines used in the present study were the immortalised trophoblast cell line HTR-8/SVneo, which has been generated by sv40 Large T Antigen transfection of first-trimester EVT cells (Graham *et al.* 1993), the choriocarcinoma cell line JEG-3 and two hybrid cell lines, based on the JEG-3-derived mutant AC1-1 cells fused with human first trimester (ACH-3P) or third trimester (AC1-M59) trophoblast cells (Gaus *et al.* 1997; Hiden *et al.* 2007). A previous study analysed the effects of OSM on MMP-2 and MMP-9 expression exclusively in HTR-8/SVneo cells, but without comparison with LIF or other cell lines (Ko *et al.* 2012). Previous comparisons have demonstrated major differences between HTR-8/SVneo cells and other trophoblast-derived cell lines and primary trophoblast cells (Bilban *et al.* 2010; Morales-Prieto *et al.* 2012; Suman and Gupta 2012). Therefore, the aim of the present study was to systematically compare the effects of LIF and OSM on the activation of major signalling pathways, as well as on invasiveness and viability, in the four aforementioned trophoblast cell lines.

Materials and methods

Cell culture and reagents

The immortalised human trophoblast cell line HTR-8/SVneo was cultured in RMPI-1640 medium (PAA Laboratories, Pasching, Austria), whereas the choriocarcinoma cell line JEG-3 and its derivatives ACH-3P and AC1-M59 were cultured in Ham F-12 medium (PAA Laboratories). The media were supplemented with 10% fetal bovine serum (Sigma, Taufkirchen, Germany), 50 U mL^{-1} penicillin and 50 µg mL^{-1} streptomycin (PAA Laboratories). Cell lines were maintained under standard conditions (37°C, 5% CO_2, humidified atmosphere). LIF was purchased from Millipore (Schwalbach, Germany) and OSM was obtained from Immunotools (Friesoythe, Germany). The STAT3 inhibitor VI, S3I-201 was obtained from Calbiochem (Darmstadt, Germany). In all experiments, the concentration of LIF and OSM used was 10 ng mL^{-1}. We chose this concentration for both cytokines in all experiments based on previous experiences with LIF (Fitzgerald *et al.* 2005; Morales-Prieto *et al.* 2013), in contrast with another study that used a twofold higher concentration of OSM (Ko *et al.* 2012).

STAT3 inhibition

The STAT3 chemical inhibitor VI, S3I-201 was used to block STAT3 phosphorylation, dimerisation and DNA binding. Cells were seeded in six-well plates, allowed to attach overnight and then treated with 200 µM STAT3 inhibitor for 24 h for subsequent Western blotting, invasion or zymography analysis.

Gel electrophoresis and Western blotting

Cells were cultured in six-well plates overnight and then stimulated with LIF or OSM. Protein extracts were prepared as follows: cells were washed with phosphate-buffered saline (PBS) containing proteinase inhibitors (5 mM β-glycerophosphate, 3 mM $MgCl_2$, 20 mg mL^{-1} aprotinin, 20 mg mL^{-1} leupeptin, 5 mg mL^{-1} pepstatin A, 647 ng mL^{-1} antipain, 10 mg mL^{-1} bestatin, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate; SERVA Electrophoresis, Heidelberg, Germany). Thereafter, cells were scraped from the bottom in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM

Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4 and 1 μg mL^{-1} leupeptin; Cell Signaling Technology, Frankfurt am Main, Germany) supplemented with protease inhibitors, followed by three freeze–thaw cycles. Protein concentrations were assessed by using the Bradford method (Sigma, Munich, Germany) and 20 μg protein extract was subjected to 9.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Resolved proteins were transferred to a nitrocellulose membrane (Hybond-P; GE Healthcare, Freiburg, Germany). Membranes were blocked with 5% defatted milk dissolved in PBS (pH 7.4) with 0.5% Tween-20, and incubated with specific antibodies (1 : 1000 dilution) diluted in NET-G buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl pH 7.4, 0.05% Triton X-100, 0.02% Gelatin) at 4°C overnight followed by incubation with a 1 : 10 000 dilution of horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody for 1 h at room temperature. Monoclonal antibodies against phosphorylated (p-) STAT3 (tyr^{705}), p-ERK1/2 ($thr^{202/204}$), β-actin or STAT3 were purchased from Cell Signaling Technology (Danvers, MA, USA).

Blots were developed using an enhanced chemiluminescence (ECL) detection kit (Millipore, Schwalbach, Germany). The intensity of Western blot bands was analysed and quantified on an MF-ChemiBis3.2 gel documentation system using Totallab TL100 software version 2006 (biostep, Jahnsdorf, Germany) and normalised against β-actin.

Cell viability assay

A CellTiter96 AQ Non-Radioactive Cell Proliferation Kit (Promega, Mannheim, Germany) was used to determine the effect of LIF or OSM on the viability of cell lines. The colour reaction of this assay is dependent on methylthiazole tetrazolium salt (MTS) reduction through NAD(P)H-dependent oxidoreductase enzymes, which correlates with cellular metabolic activity. Briefly, 5000 cells were plated in 96-well microtitre plates and cultured in medium containing 10% fetal bovine serum (100 μL per well) in the presence or absence of LIF or OSM. After 24 h incubation, 20 μL MTS solution was added into each well and samples were incubated for a further 1–4 h. Absorbance was read at 490 nm on a 96-well plate reader.

Cell invasion assay

The *in vitro* invasion assay was performed in 24-well inserts with porous membranes (8-μm pore size; Millipore, Schwalbach, Germany). Briefly, 30 μL diluted Matrigel (1 : 3) was added to the entire surface of the membrane. Coated inserts were incubated at 37°C for 30 min to allow the Matrigel to solidify. Suspensions of 1×10^5 cells in 200 μL were transferred to the inserts precoated with Matrigel (BD Biosciences, Heidelberg, Germany) and incubated with or without LIF or OSM in the upper and lower chambers. After 24 h incubation at 37°C in a 5% CO_2 atmosphere, cells in the upper chamber were removed with a cotton swab, whereas invading cells were fixed with chilled 80% ethanol and stained with 0.1% crystal violet. After washing, cells were eluted by adding 200 μL of 1% acetic acid. Colorimetric absorbance was detected at 570 nm and normalised against untreated cells.

STAT3 DNA binding assay

STAT3 nuclear activation was quantified by using a TransAM STAT family kit (Active Motif, La Hulpe, Belgium) according to the manufacturer's protocol. Briefly, cells were transferred to six-well plates and treated with LIF (10 ng mL^{-1}) or OSM (10 ng mL^{-1}) for 4 h. Nuclear protein fractions were extracted using a Nuclear Extract Kit (Active Motif). Then, 5 μg nuclear extract was incubated for 1 h in 96-well plates coated with oligonucleotides containing the STAT3 consensus binding site (5′-TTCCCGGAA-3′). Active STAT3 in the nuclear extract bound to the oligonucleotides was detected by a STAT3-specific antibody and an HRP-conjugated secondary antibody. After addition of the developing solution, absorbance was measured at 450 nm, corrected to blank and normalised against untreated cells.

Zymography

After 24 h incubation with the STAT3 inhibitor, cells were incubated with or without LIF in serum-free medium for a further 48 h. Finally, cells and supernatants were collected separately. Conditioned media were concentrated by using Amicon ultra-4 centrifugal filter devices (Millipore). Cells were lysed with lysis buffer on ice and then sonicated for 1 min. Protein concentrations in the conditioned supernatants were determined by the Bradford protein assay (Sigma) and equal amounts of protein (10 μg) were subjected to gel electrophoresis. Equal amounts of protein were incubated with 5× non-reducing loading buffer for 10 min at room temperature and electrophoresed on 9% acrylamide gels containing 0.1% w/v gelatine under non-reducing conditions. After two washing steps for 30 min with 2.5% Triton X-100 solution, gels were incubated for 18 h at 37°C in developing buffer (50 mM Tris, 200 mM NaCl, 10 mM $CaCl_2$, pH 7.4). The divalent metal cation triggers enzymatic activity of otherwise non-active pro-MMPs. Gels were stained with Coomassie brilliant blue R-250 (0.1% w/v). The digested areas appear clear on a blue background, correlating with the protein concentration of the gelatinases, which were quantified on an MF-ChemiBis3.2 gel documentation system using Totallab TL100 software (biostep).

Statistical analyses

Western blots and zymography were repeated at least three times. DNA-binding, invasion and viability assays were performed independently at least three times and each experiment comprised three independent replicates. Student's *t*-tests were used for statistical analyses. Two-sided $P \leq 0.05$ was considered significant.

Results

STAT3 and ERK1/2 expression

STAT3 and ERK1/2 were constitutively expressed at different levels in all cell lines tested. Although ERK2 expression was

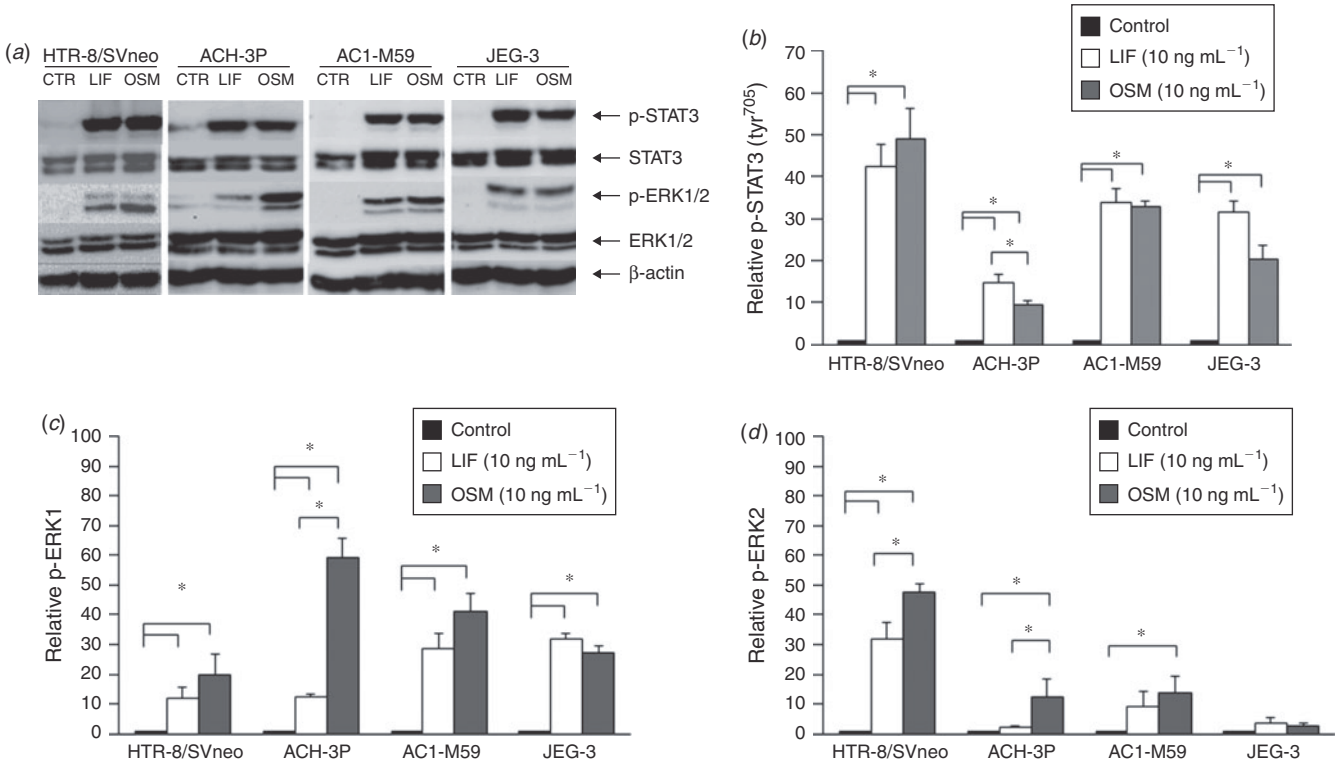

Fig. 1. Expression and phosphorylation of signal transducer and activator of transcription (STAT) 3 and extracellular signal-regulated kinase (ERK) 1/2 in trophoblast cell lines. Cells were serum starved for 2 h and subsequently stimulated with leukaemia inhibitory factor (LIF; 10 ng mL^{-1}) or oncostatin M (OSM; 10 ng mL^{-1}) for 15 min. (*a*) Representative Western blot membranes for phosphorylated (p-) STAT3 (tyr^{705}), total STAT3, p-ERK1/2 (thr$^{202/204}$), total ERK1/2 and β-actin. Band intensities were assessed by densitometry and ratios between phosphorylated and respective total protein calculated. Control values were given a value of 1. (*b–d*) Relative expression of p-STAT3 (*b*), p-ERK1 (*c*) and p-ERK2 (*d*). Data are the mean ± s.e.m. of three independent experiments. *$P < 0.05$ (Student's *t*-test).

higher than that of ERK1 in HTR-8/SVneo cells, in all choriocarcinoma-derived cell lines ERK1 expression was higher than that of ERK2 (Fig. 1*a*; densitometry results not shown).

Effects of LIF and OSM on STAT3 and ERK1/2 phosphorylation

Slight constitutive phosphorylation of STAT3 (tyr^{705}) was detectable only in HTR-8/SVneo and ACH-3P cells. LIF and OSM induced phosphorylation of STAT3 (tyr^{705}) and ERK1/2 (thr$^{202/204}$) in all analysed cell lines, but at different intensities (Fig. 1*a*). LIF and OSM induced stronger STAT3 phosphorylation in HTR-8/SVneo cells than in ACH-3P, AC1-M59 and JEG-3 cells (Fig. 1*b*). The effects of LIF and OSM did not differ significantly, except in ACH-3P cells, where LIF induced slightly but significantly stronger STAT3 phosphorylation (Fig. 1*b*).

In general, LIF and OSM induced stronger phosphorylation of ERK1 than ERK 2 in all choriocarcinoma-derived cell lines, but stronger phosphorylation of ERK2 than ERK1 in HTR-8/SVneo cells (Fig. 1*c*, *d*). The effects of OSM and LIF on ERK1/2 phosphorylation were similar in AC1-M59 and JEG-3 cells. The effect of OSM was significantly stronger than that of LIF on ERK1 phosphorylation in ACH-3P cells (Fig. 1*c*). The effect of OSM was also significantly stronger on ERK2 phosphorylation in ACH-3P and HTR-8/SVneo cells (Fig. 1*d*).

Effects of LIF and OSM on STAT3 DNA-binding capacity

Stimulation of all four cell lines with LIF and OSM significantly increased STAT3 DNA-binding activity compared with untreated cells (Fig. 2). In the JEG-3 derivates, LIF and OSM induced similar levels of STAT3 DNA-binding activity, whereas in HTR-8/SVneo cells LIF induced STAT3 DNA-binding activity significantly stronger than OSM. In JEG-3 cells, LIF and OSM induced the highest increase in STAT3 DNA-binding activity (4.7- and 4.1-fold increases, respectively). ACH-3P, HTR-8/SVneo and AC1-M59 cells were less responsive in this respect. No further increase in STAT3 DNA binding was observed when using 20 ng mL^{-1} OSM in any of the cell lines tested (data not shown).

Effects of LIF and OSM on cell invasion and viability

In our hands, neither LIF nor OSM significantly affected the viability of any of the cell lines tested (data not shown). To investigate the effects of LIF and OSM on invasiveness, cells were incubated in the presence or absence of LIF (10 ng mL^{-1})

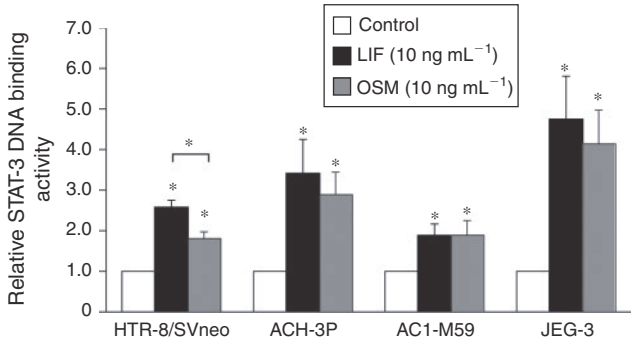

Fig. 2. DNA binding activity of signal transducer and activator of transcription (STAT) 3 upon stimulation with leukaemia inhibitory factor (LIF) or oncostatin M (OSM). Trophoblast cell lines were starved for 2 h and subsequently treated in presence or absence of LIF (10 ng mL^{-1}) or OSM (10 ng mL^{-1}) for a further 4 h. The nuclear fraction was extracted and analysed for STAT3 DNA-binding capacity by using an ELISA-based colorimetric assay kit. Samples were measured at an optical density of 450 nm. Control values were given a value of 1; values from other groups are expressed relative to the control group. Data are the mean ± s.e.m. of nine independent experiments. *$P < 0.05$ compared with control or as indicated (Student's *t*-test).

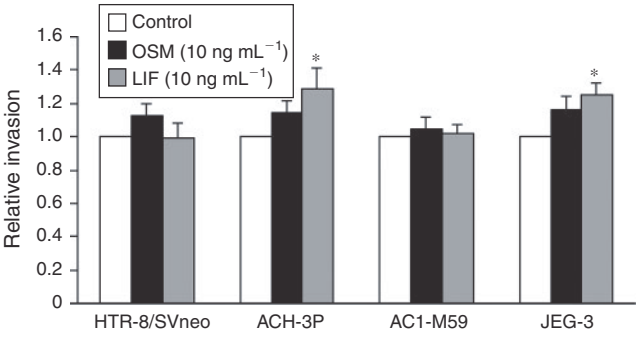

Fig. 3. Effects of leukaemia inhibitory factor (LIF) and oncostatin M (OSM) on the invasiveness of trophoblast cell lines, assessed with a Matrigel invasion assay. Cells were incubated with or without LIF (10 ng mL^{-1}) or OSM (10 ng mL^{-1}) for 24 h. Staining of invaded cells was assessed by measuring absorbance on a plate reader. Control values were given a value of 1; values from other groups are expressed relative to the control group. Data are the mean ± s.e.m. of three independent experiments. *$P < 0.05$ compared with control (Student's *t*-test).

or OSM (10 ng mL^{-1}) for 24 h in a Matrigel invasion assay. Compared with their respective unstimulated controls, LIF significantly enhanced invasiveness in ACH-3P (by 29%) and JEG-3 (by 25%) cells, but not in HTR-8/SVneo or AC1-M59 cells. OSM induced a slight (and non-significant) increase in invasiveness in HTR-8/SVneo, ACH-3P and JEG-3 cells (Fig. 3).

Effects of STAT3 inhibition on invasion

Twenty-four hour treatment with the STAT3 inhibitor decreased LIF-induced STAT3 expression and activation by approximately 60% and 80% in ACH-3P and JEG-3 cells, respectively, as confirmed by Western blotting (Fig. 4*a*, *b*). Inhibition of STAT3 phosphorylation resulted in a significant reduction in ACH-3P and JEG-3 invasion (by 25%; Fig. 4*c*), but had no effect on cell viability (Fig. 4*d*).

Effects of blocking STAT3 phosphorylation on MMP-2 and MMP-9 activity

Conditioned medium and lysates from ACH-3P and JEG-3 cells were obtained after 24 h treatment with the STAT3 inhibitor, followed by 48 h with or without LIF. Protein levels of pro-MMP-2 (72 kDa), active MMP-2 (62 kDa), pro-MMP-9 (92 kDa) and active MMP-9 (82 kDa) were detected by gelatine zymography (Fig. 5*a*). Stimulation of ACH-3P and JEG-3 cells with LIF significantly increased pro-MMP-2 and active MMP-2 levels in conditioned medium from both cell lines, and pro-MMP-9 levels only in conditioned medium from JEG-3 cells. The pro-MMP-2 levels in cell lysates were not affected by LIF stimulation (Fig. 5*b*). Inhibition of STAT3 phosphorylation significantly decreased pro-MMP-2 and MMP-2 protein levels in conditioned media from ACH-3P and JEG-3 cells, and pro-MMP-9 protein levels in conditioned medium from JEG-3 cells. Further administration of LIF could not rescue the effects of the STAT3 inhibitor. Protein levels of pro-MMP-2 increased significantly in lysates of both cell lines after treatment with the STAT3 inhibitor (Fig. 5*b*).

Discussion

The purpose of the present study was to assess and compare the effects of the IL-6-type cytokine family members LIF and OSM on different trophoblast cell lines. Some effects of LIF, mainly on JEG-3 and HTR-8/SVneo cells, have been published previously and have been repeated and confirmed here as a comparison for the effect of OSM. Stimulation with LIF and OSM induced STAT3 (tyr^{705}) and ERK1/2 phosphorylation and STAT3 DNA binding in all cell lines tested. These results suggest that LIF and OSM transduce their effects via their shared common receptor unit gp130, which is known to activate these pathways (Plun-Favreau *et al.* 2003). LIF and OSM induced similar levels of STAT3 phosphorylation in the cell lines tested, except for HTR-8/SVneo cells, where the effects appear to be slightly stronger (effects summarised in Table 1).

In our hands, LIF and OSM had no significant effect on cell viability in any of the cell lines tested, as assessed by the MTS assay, which analyses metabolic activity in a cell culture. These results after 24 h cell culture seem to disagree with previously published results from our group, whereby 10 ng mL^{-1} LIF was found to increase the proliferation in JEG-3 cells as assessed by cell counting after 72 h (Fitzgerald *et al.* 2005). However, the time frames differ between the two studies and the two methods used to determine viability work with different parameters, which may lead to diverse results, as shown previously (Strober 2001; Berridge *et al.* 2005; Wang *et al.* 2010; Hoskins *et al.* 2012). Results derived from the MTS assay serve as a control to exclude the influence of cell viability on invasion.

LIF and OSM triggered the phosphorylation of ERK1 and ERK2. The expression pattern of p-ERK1/2 in HTR-8/SVneo cells differed from that in choriocarcinoma-derived cell lines.

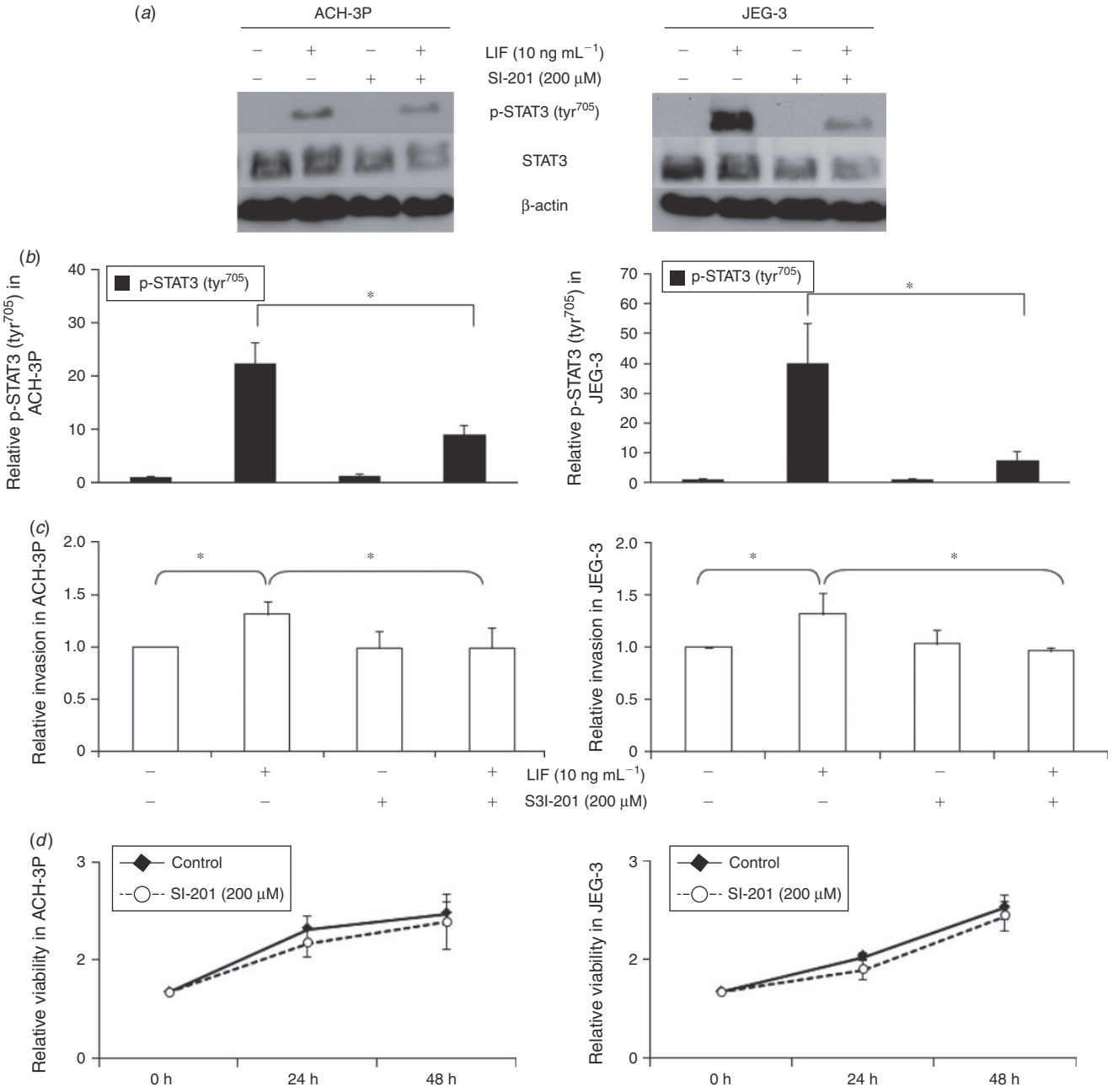

Fig. 4. Effects of signal transducer and activator of transcription (STAT) 3 inhibition on invasion and viability of ACH-3P and JEG-3 cells. ACH-3P cells and JEG-3 cells were pretreated or not with a STAT3 inhibitor (200 μM) for 24 h. Subsequently, STAT3 phosphorylation (tyr^{705}) was analysed by Western blotting, and Matrigel invasion and methylthiazole tetrazolium salt (MTS) viability assays were performed. (*a*) Representative Western blots checking STAT3 inhibitor efficacy. Before lysis, cells were stimulated with leukaemia inhibitory factor (LIF) for 15 min to induce STAT3 phosphorylation. (*b*) Band intensities were assessed by densitometry. Applied concentrations as in (*a*). (*c*) For assessment of invasiveness, cells were placed in the upper chamber of Matrigel coated wells on a transwell plate and exposed or not to LIF (10 ng mL^{-1}) for 24 h. The invading cells were stained and intensity was quantified on a plate reader. (*d*) For assessment of viability, cells were cultured in 96-well plates for a further 48 h. Relative viability was assessed using an MTS assay and colorimetric analysis on a plate reader. Data are the mean ± s.e.m. of three independent Western blotting experiments and nine viability and invasion assays. *$P < 0.05$ (Student's *t*-test).

After treatment with LIF or OSM, phosphorylation of ERK2 was higher than that of ERK1 in HTR-8/SVneo cells, whereas in the choriocarcinoma-derived cell lines levels of p-ERK1 were higher. Only in ACH-3P cells did OSM induce a more intensive phosphorylation of ERK1/2 than LIF. Previously, we reported that inhibition of ERK by U0126 significantly decreased JEG-3 and HTR-8/SVneo proliferation and increased JEG-3 invasiveness (Prakash *et al.* 2011; Morales-Prieto *et al.* 2013).

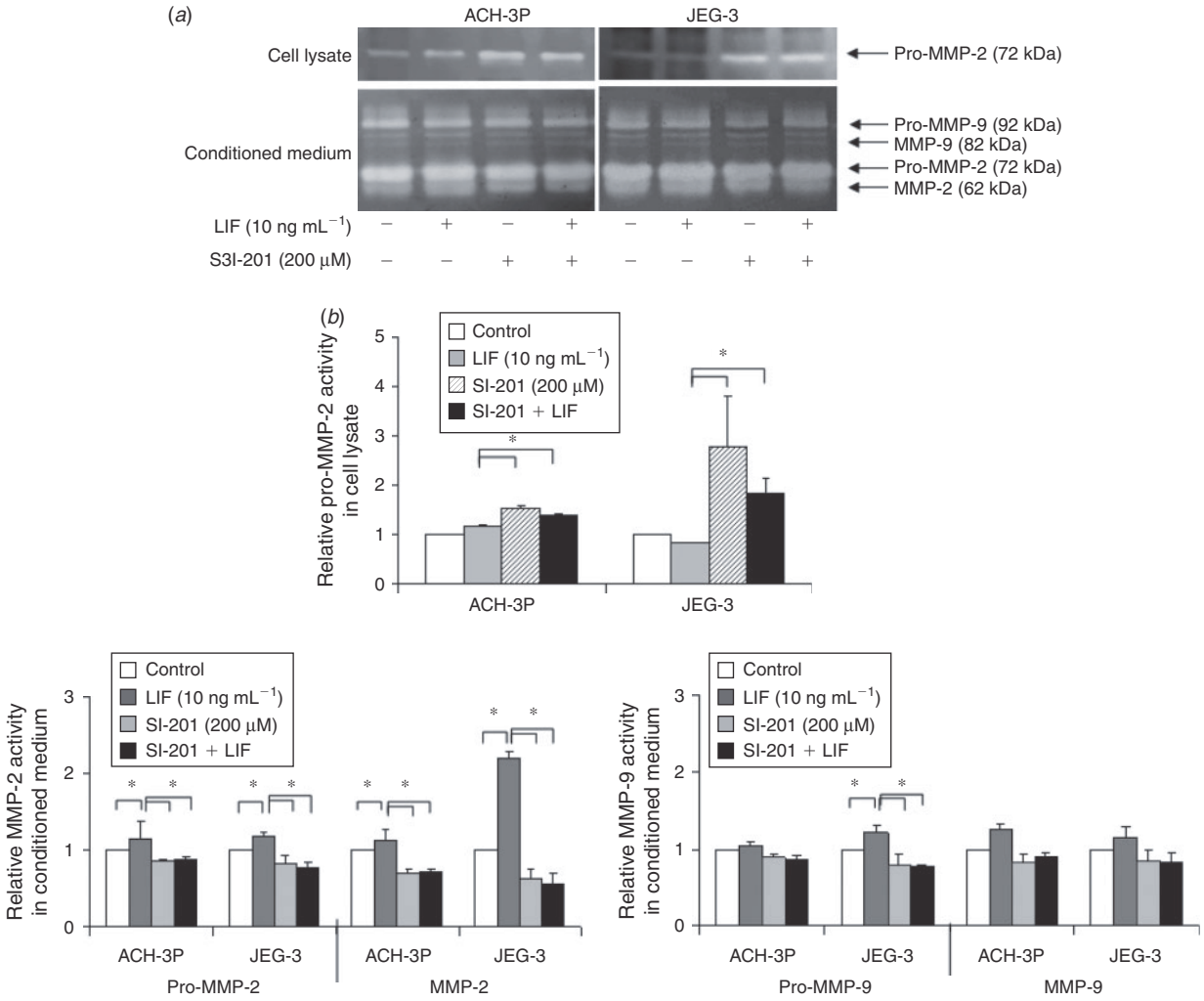

Fig. 5. Effects of signal transducer and activator of transcription (STAT) 3 inhibition on matrix metalloproteinase (MMP)-2 and MMP-9 activity in conditioned medium and cell lysates. ACH-3P and JEG-3 cells were pretreated with or without the STAT3 inhibitor (200 μM) for 24 h, followed by stimulation with or without leukaemia inhibitory factor (LIF; 10 ng mL^{-1}) in serum-free medium for 48 h. Media and cell lysates were collected, concentrated and (*a*) MMP-2 and MMP-9 activity was assessed by gelatin zymography. (*b*) Densitometric analyses of zymography bands. The intensity of control samples was given a value of 1; values from other groups are expressed relative to the control group. Data are the mean ± s.e.m. of three independent assays. *$P < 0.05$ (Student's *t*-test).

Table 1. Summary of the effects of leukaemia inhibitory factor and oncostatin M on trophoblast cells

↑, increased; ↑↑, significantly increased; n.c., not changed; p-, phosphorylated; STAT3, signal transducer and activator of transcription; ERK, extracellular signal-regulated kinase; LIF, leukaemia inhibitory factor; OSM, oncostatin M

Cell line	p-STAT3		p-ERK1		p-ERK2		STAT3 DNA binding		Viability		Invasion	
	LIF	OSM	LIF	OSM	LIF	OSM	LIF	OSM	LIF	OSM	LIF	OSM
HTR-8/SVneo	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	n.c.	n.c.	n.c.	↑
ACH-3P	↑↑	↑↑	↑↑	↑↑	↑	↑↑	↑↑	↑↑	n.c.	n.c.	↑↑	↑
AC1-M59	↑↑	↑↑	↑↑	↑↑	↑	↑↑	↑↑	↑↑	n.c.	n.c.	n.c.	n.c.
JEG-3	↑↑	↑↑	↑↑	↑↑	↑	↑	↑↑	↑↑	n.c.	n.c.	↑↑	↑

Invasion of trophoblast cells into the decidua is essential for successful implantation and placentation (Knöfler and Pollheimer 2012). Several factors control this process, including cytokines, adhesion molecules and ECM-degrading enzymes (Goldman-Wohl and Yagel 2002; Knöfler and Pollheimer 2012). MMPs are not only involved in ECM degradation under pathological conditions, but are also associated with trophoblast invasion in pregnancy, especially MMP-2 and MMP-9, which are mainly produced by EVT and villous cytotrophoblast, respectively (Cohen *et al.* 2006; Demir-Weusten *et al.* 2007). Recently, it was reported that 20 ng mL^{-1} OSM stimulated the invasion of HTR-8/SVneo cells by increasing MMP-2 and MMP-9 levels (Ko *et al.* 2012). The results of the present study indicate that 10 ng mL^{-1} OSM slightly increases the invasiveness of all cells lines, whereas 10 ng mL^{-1} LIF induces a significant increase in the invasiveness of ACH-3P and JEG-3 cells.

In the present study, the invasiveness of HTR-8/SVneo was not significantly induced by either LIF or OSM. It can be argued that the concentrations used in the present study were lower than in other studies; for example, Suman *et al.* (2013) found that 50 ng mL^{-1} LIF increased the invasion of HTR-8/SVneo cells. Nevertheless, the concentrations used in the present study enhanced the invasiveness of the choriocarcinoma-derived cell lines JEG-3 and ACH-3P, which is in line with a previous report (Morales-Prieto *et al.* 2013). Conversely, it can be argued that the origin of HTR-8/SVneo cells may be responsible for this cellular response. The HTR-8/SVneo cell line is an immortalised cell line exhibiting a highly invasive phenotype that may not be further enhanced by comparatively low concentrations of LIF and OSM.

The release of MMP-2 and MMP-9 from trophoblastic cells, including from first-trimester EVT (Tapia *et al.* 2008) and HTR-8/SVneo cells (Busch *et al.* 2009), has been demonstrated previously. After 24 h stimulation of EVT with LIF, Tapia *et al.* (2008) did not find any increases in MMP release, whereas in the present study LIF induced significant MMP release by ACH-3P and JEG-3 cells after 48 h. In lysates from ACH-3P and JEG-3 cells, pro-MMP-2 protein levels increased after STAT3 inhibition, whereas active MMP-2 was not detectable. This means that the non-active form accumulates in the cells when STAT3 is inhibited. Simultaneously, LIF-induced MMP-2 and MMP-9 release into the medium was significantly reduced in both cell lines, although MMP-9 isoforms were not detectable in cell lysates. These results are in line with previous studies in other cell models demonstrating that STAT3 seems to be involved in the LIF-induced MMP release; for example, in fibroblasts it activates MMP-9 release (Wang *et al.* 2007). In ovarian cancer cells, inhibition of STAT3 led to a decrease in MMP-2 activity in cell culture supernatant (Seo *et al.* 2012). Additional LIF could not reverse the effects of STAT3 inhibition on MMP-2 and MMP-9 expression. These results imply that LIF enhances MMP-2 and MMP-9 secretion exclusively through STAT3.

Conclusion

In trophoblast cell lines, OSM shares STAT3 and ERK1/2 signalling with LIF, as well as the capacity to induce invasiveness through activation of MMP-2 and MMP-9. Physiologically, *in utero*, these cytokines may act synergistically or cross-compensate for deficiencies. Their functions differ slightly, which indicates the involvement of further intracellular cascades, potentially activated through the different gp130 receptor subunits.

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4.2 Part II: miRNAs regulate trophoblast cell behavior

Publication lists

4.2.1 **MicroRNA expression profiles of trophoblastic cells**

Morales-Prieto DM, **Chaiwangyen W**, Ospina-Prieto S, Schneider U, Herrmann J, Gruhn B, Markert UR. *Placenta*. 2012 Sep;33(9):725-34

Trophoblast cell lines and primary trophoblast cells express different pattern of major placenta related miRNAs including C19MC, C14MC and miR-371-3 clusters.

4.2.2 **Pregnancy-associated miRNA-clusters**

Morales-Prieto DM, Ospina-Prieto S, **Chaiwangyen W**, Schoenleben M, Markert UR. *J Reprod Immunol*. 2013 Mar;97(1):51-61

In human, C19MC, C14MC and miR-371-3 clusters are predominately express during pregnancy. These clusters can be detected in maternal blood and potential as noninvasive biomarkers for pregnancy complications.

4.2.3 **Elsevier Trophoblast Research Award Lecture: Origin, evolution and future of placenta miRNAs**

Morales-Prieto DM, Ospina-Prieto S, Schmidt A, **Chaiwangyen W,** Markert UR. *Placenta*. 2014 Feb;35 Suppl:S39-45

The human placenta specific miRNAs: C19MC, C14MC and miR-371-3 clusters are connected to evolution similar to placenta development. Dysregulation of these miRNAs may be associated with pregnancy related disorders. Release of these miRNAs into maternal circulation may provide novel powerful clinical biomarkers in diagnosis of pregnancy related disorders.

4.2.4 **miR-21 regulates trophoblast cell functions by targeting phosphatase and tensin homologue (PTEN) and programmed cell death 4 (PDCD4)**

Chaiwangyen W, Ospina-Prieto S, Morales-Prieto DM, Mary S, Schleussner E, Markert UR. *RNA biology* (Submitted)

miR-21 is highly expressed in trophoblast cells and involved in regulating cell proliferation, migration, invasion and apoptosis in trophoblastic cell lines by targeting PTEN and PDCD4.

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MicroRNA expression profiles of trophoblastic cells

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ABSTRACT

Background: MicroRNAs (miRNAs) are small single-stranded RNA molecules working as post-transcriptional modulators of gene expression. Trophoblast cells are a heterogenous group of fetal cells forming the feto–maternal interface and displaying a wide spectrum of functions. The regulation of their behavior may partly underly the control through miRNAs. Therefore, we aimed to compare the miRNA profile of primary first and third trimester trophoblast cells with that of different trophoblastic cell lines.
Material and methods: Total RNA was obtained from isolated cytotrophoblast cells from healthy term and first trimester placentae and the cell lines HTR-8/SVneo (immortalized trophoblast cells), JEG-3 (choriocarcinoma), ACH-3P and AC1-M59, which are choriocarcinoma cells fused with first and third trimester trophoblast cells, respectively. The expression level of 762 different miRNAs was quantitatively analyzed by using a TaqMan Human MicroRNA Array. For testing the reproducibility of the array technique, the expression of 9 selected miRNAs has been re-analyzed by individual qPCR.
Results: The analyzed cell types share many similar patterns of miRNAs, but are significantly distinct in the expression of three miRNA clusters: chromosome 19 miRNA cluster (C19MC; containing 54 different miRNAs), C14MC (34 miRNAs) and a minor cluster (miRNA-371 to miRNA-373 cluster), also located on chromosome 19. Expression of miRNAs within C19MC increases significantly from first to third trimester trophoblast while that of C14MC members decreases. MiRNAs within the miR-371-3 cluster augment slightly. C19MC and the miR-371-3 cluster are not expressed by HTR-8/SVneo cells whilst C14MC is almost not detectable in the choriocarcinoma-derived cell lines complete array data available at NCBI Gene Expression Omnibus accession number GSE32346: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE32346). Beside the miRNAs within the mentioned clusters, further 27 miRNAs are differentially expressed (>100 fold) between term and first trimester trophoblast cells. The placenta-specific miRNAs miR-141 and miR-21 as well as let-7g are expressed in all tested cells with the highest expression in primary trophoblast cells.
Conclusion: Primary first trimester and term trophoblast cells and trophoblastic cell lines display major differences in their miRNA fingerprints which may be involved in their different behavior and characteristics.

1. Introduction

Since the discovery of the first microRNA *lin-4* in 1993 [1], the study of microRNAs (miRNAs) has generated great interest due to their vast potential in the regulation of protein-coding genes. MiRNAs are highly conserved sequences of single-stranded RNA (~19–22 nt) which repress gene expression by a mechanism involving the RNA interference pathway [2]. Depending on the complementary grade between the miRNA and its mRNA target, the pathway results in inhibition of translation, or cleavage of the target mRNA, when partially or fully complimentary, respectively [3]. This characteristic allows targeting of several genes simultaneously and therefore, it is expected that 30% of the human genome may be regulated by miRNAs [4].

Remarkably, miRNA genes are frequently located at fragile sites and cancer-related genomic regions [5], and tend to be organized into clusters suggesting that miRNAs belonging to a same cluster

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are likely to have similar functions and be regulated by the same promoter and in the same transcriptional orientation [6,7]. The analysis of the miRNA signature (miRNome) in normal human tissues revealed some universally expressed miRNAs but also several groups of miRNAs exclusively or preferentially expressed in a tissue-specific manner [8]. Likewise, the miRNA expression signature is frequently altered in cancer [9,10], and can be used to distinguish between cancer and normal tissues [11,12] or even to identify poorly differentiated tumors [13].

Recent reports have described two large miRNA clusters expressed in placenta: The chromosome 19 miRNA cluster (C19MC), which maps to chromosome 19q13.41 and comprises 54 predicted miRNAs, 43 of which have been already cloned and sequenced (reviewed in Ref. [3]); and the C14MC located in the 14q32 domain and which contains 34 miRNAs [14]. C19MC is exclusively found in primates while C14MC appears to be conserved among all eutherian species [15]. Both are encoded within imprinted domains: C19MC is expressed from the paternally inherited chromosome whilst C14MC is expressed from the maternally inherited chromosome [14,15]. Imprinted genes are known to be involved in human embryonic development and to play important roles in the regulation of cellular differentiation and fate [16].

The signature of miRNAs in trophoblast cells is largely unknown. Initial investigations identified some miRNAs specifically expressed in placenta tissue and released into maternal plasma [17]. Further analyses of the expression of small RNAs in placenta by small RNA library sequencing confirmed that most placenta-specific miRNAs were linked to the C19MC cluster and demonstrated that villous trophoblast cells express such miRNAs [18]. Recent reports have quantitatively analyzed the expression of up to 820 miRNAs in placenta tissue samples collected in the first or third trimester [19,20]. Interestingly, the concentration of pregnancy-associated miRNAs increased throughout pregnancy [19] and was altered in placentas from pregnancies with preeclampsia or preterm labor [20]. These results suggest miRNAs as potential serum markers for the normal function of the placenta. However, little is known about the cellular origin of these placental miRNAs.

The study of the miRNome of isolated trophoblast cells is restricted by the limitations associated with primary cells such as relatively low number of isolated cells, short lifespan or lack of proliferation *in vitro* [21]. Therefore, during the last three decades, several trophoblastic cell lines have been established attempting to resemble primary trophoblasts and avoiding the limitations of isolation. Two main methodologies have been used: Introduction of the gene encoding simian virus 40 large T (sv40T) antigen [22] or establishment of human choriocarcinoma cell lines [23]. Therefore, the different genetic background and the methods used for immortalization should be taken into consideration for interpretation and discussion of results obtained from the respective cell line.

To our knowledge, there is a very limited number of publications on miRNA expression profiles in trophoblastic cell lines, or their comparison with primary isolated trophoblast cells [24]. To overcome this lack of knowledge, we assessed the miRNA expression patterns of four cell lines and isolated trophoblast cells from first and third trimester placentae. We included the immortalized human first trimester trophoblast cell line HTR-8/SVneo [22], the choriocarcinoma cell line JEG-3 and the two hybrids cell lines, ACH-3P and AC1-M59, which resulted of fusion of the AC-1 choriocarcinoma cell line with first and third trimester isolated trophoblast cells, respectively [23,25].

For confirmation of array results, expression of 9 individual miRNAs was assessed by qPCR. Instead of random choice, we selected five miRNAs representing the C19MC (miR-518a-5p and miR-519e), the C14MC (miR-411 and miR-539) and the miR-371-3 cluster (miR-373); as well as four miRNAs that have been previously described to be expressed in placenta or tumors: miR-9, miR-21, miR-141, and let-7g [3,14,17,18,26].

By fingerprinting miRNA gene expression we aimed to contribute to better understanding of differences and resemblances of these frequently used cell lines and primary trophoblast cells. Concluding from our observations, the above mentioned cluster C14MC and C19MC may play key roles in regulating their phenotypical and functional diversity.

2. Materials and methods

2.1. Cell lines

Four cell lines were investigated in this study: the immortalized extravillous first trimester trophoblast cell line HTR-8/SVneo (kind gift from CH Graham, Kingston Canada) [22], the choriocarcinoma cell line JEG-3 (DSMZ, Braunschweig, Germany), and two hybrids of AC-1 choriocarcinoma cells (derived from JEG-3 by mutagenesis) with human first and third trimester trophoblast cells, ACH-3P and AC1-M59 cells, respectively (kind gift from G Desoye, Graz, Austria) [23,25,27].

2.2. Cell culture

Cell cultures were performed at 10^6 cells/175 cm^2 flask, and maintained under standard conditions (37 °C, 5% CO_2, humid atmosphere) in Ham's F-12 Nutrient Mixture with L-glutamine (GIBCO, Paisley, UK) or RPMI Medium (GIBCO) (HTR-8/SVneo cells) supplemented with 10% heat-inactivated fetal calf serum (FCS; GIBCO) and 1% penicillin/streptomycin antibiotic solution (GIBCO).

2.3. Primary trophoblast isolation protocol

Healthy term placentae were obtained from the Department of Obstetrics, University Hospital, Jena, Germany. First trimester placentae have been received after elective pregnancy termination at week 8–12 from the Department of Gynecology of the Robert-Koch-Hospital, Apolda, Germany. Trophoblast isolation was performed using a modified Kliman method as described in detail by Stenqvist et al. [28]. Briefly, placental tissue was physically disaggregated and enzymatically digested for 30 min. After washing, isolated cells were applied on a density gradient (Percoll, Pharmacia, Sweden) and the fraction retained within the layer of 25% Percoll was collected and washed. For depletion of non-trophoblastic cells, Dynabeads coated with anti-CD45 and anti-CD82 antibodies (Life Technologies, Darmstadt, Germany) were used. Before use for RNA isolation, isolated cells have been cultured in F-12 medium (GIBCO) supplemented with 10% FCS for two days, and remaining non-adherent cells have been removed. Adherent cells have been checked for purity by flow cytometry using anti-EGF-receptor, anti-cytokeratin-7, and anti-HLA-G antibodies. The levels of impurity (cells negative for mentioned markers) did not exceed 10%.

2.4. RNA isolation and array analysis

Cells were seeded in 12-well plates, allowed to attach overnight and serum deprived for at least 2 h. Total RNA was isolated by using a mirVana isolation kit (Life Technologies, Darmstadt, Germany), according to the manufacturer's protocol. Thereafter, 100 ng of total RNA containing miRNAs was reverse transcribed using the specific Megaplex RT primers (Life Technologies) followed by a pre-amplification of the obtained cDNAs. Finally, the expression level of 762 different miRNAs was performed using the TaqMan® Array Human MicroRNA A + B Cards Set v3.0 (Life Technologies) on a 7900 Real Time PCR System (Applied Biosystems). Card A includes miRNAs, which tend to be defined and broadly or highly expressed, whilst those of card B are less extensively studied or expressed at low levels (suppliers product description). Experimental data were analyzed by DataAssist v3.0 (Life Technologies) using RNU48 and RNU44 as endogenous controls. The amplification efficiency for TaqMan gene expression assays has been tested and described by the manufacturer in detail (Application Note, Applied Biosystems). It reaches 100% (±10%). Due to software settings, results from card A and card B had to be analyzed separately and are displayed as heatmaps from unsupervised hierarchical clustering of all miRNAs and all individual samples. The arrays were repeated independently twice for ACH-3P, AC1-M59 cells and HTR-8/SVneo, and three times for JEG-3 and trophoblast cells.

2.5. Real-time quantitative RT-PCR

The expression levels of five miRNAs (miR-518a-5p, miR-519e, miR-373, miR-411, miR-539) representing three different miRNA clusters (C19MC, cluster miR-371-3, C14MC) were confirmed by applying individual TaqMan miRNA Assays (Applied Biosystems, Foster City, CA, USA) according to the protocol provided by the supplier. Additionally, the expression of another set of 4 miRNAs (miR-9, miR-21, miR-141, let-7g), which are known to correlate with tumor-grade, to be implicated

in pregnancy or to be related with members of the intracellular signaling cascade of LIF was confirmed by use of the same method [26]. Total RNA was isolated by using a mirVana isolation kit (Life Technologies). RNA purity was assessed by the ratio of spectrophotometric absorbance at 260 and 280 nm (A260/280 nm) on a NanoDrop ND-1000 (NanoDrop Inc, Wilmington, DE USA). Reverse transcription was performed with miRNA specific stem-loop RT primers and TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems), followed by qRT-PCR using specific TaqMan Assays and TaqMan Universal PCR Master Mix. All reactions were run in duplicates including no-template controls in 96-well plates on a 7300 Real Time PCR System (Applied Biosystems). Fold changes were calculated by the formula $2^{-\Delta\Delta Ct}$ relative to the expression in primary first trimester trophoblast cells. RNU48 has been used as endogenous control, which has provided the highest stability and expression level

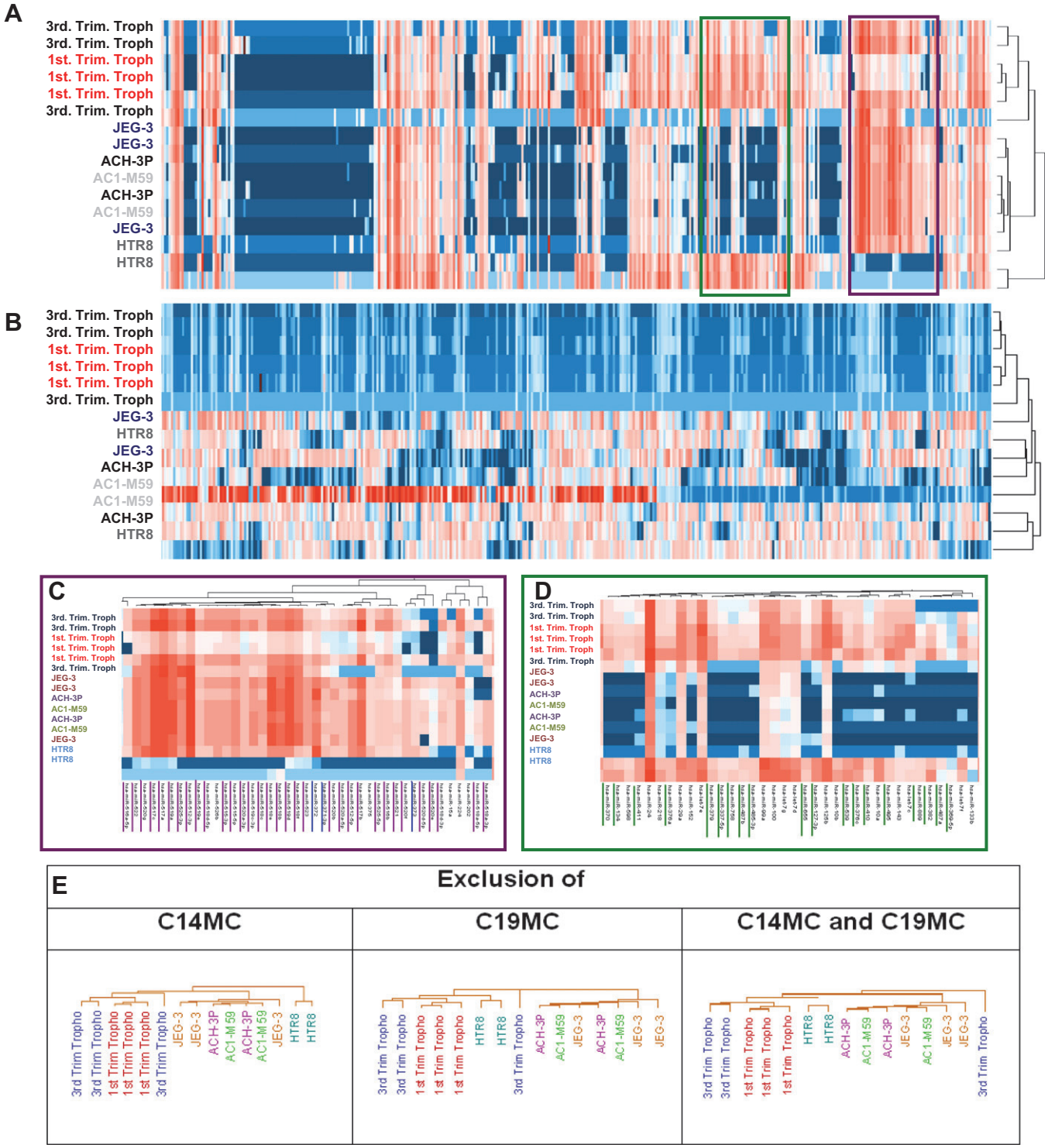

Fig. 1. Unsupervised hierarchical clustering analysis of miRNAs expression profiles of all individually analyzed samples and miRNAs. The level (Ct-value) of miRNA expression is color-coded. Red: higher miRNA expression; blue: lower miRNA expression. A) and B) represent the 381-containing miRNA assays A and B, respectively. C) and D) Zoom into the boxes marked in A), which display expression of miRNAs with highly different expression between HTR-8/SVneo and the other cells. MiRNAs which belong to C19MC are underlined in purple, to C14MC in green and to cluster 371–373 in blue. E) Dendograms of the unsupervised hierarchical clustering of Assay A after exclusion of data from the leading clusters. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in our model compared with RNU44 and MammU6. In our analyses ($n = 18$) the Ct-values for RNU48 varied between 20.81 and 23.72 (mean 21.76; SD 0.72). The experiments were repeated independently three times and differences in the quantified gene expression were statistically assessed by using a Student's *t*-test and considered statistically significant when $p < 0.05$.

3. Results

3.1. Expression profiles of miRNAs in isolated trophoblast cells and cell lines

We assessed the complete (miRBase v13.0) microRNA expression profile of the four trophoblastic cell lines HTR-8/SVneo, JEG-3, AC1-M59 and ACH-3P as well as that of trophoblast cells isolated from first and third trimester placentae (complete array data are accessible trough GEO Series accession number GSE32346; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE32346).

The detection of the total of 762 miRNAs was performed on two different array cards A and B containing 381 miRNAs each. Around 65% of the miRNAs on card A were notably expressed (Ct < 35.0) in any analyzed cell type, but only approximately 35% of those on card B (Fig. 1A–B).

Unsupervised cluster analysis of all Ct-values revealed that in both arrays (card A and B) isolated trophoblast cluster closely together in neighbored branches of the dendogram, while cell lines were more distant. This analysis was also able to discriminate between first and third trimester samples, except for one sample of third trimester trophoblast cells which had a generally low miRNA expression. The analysis of card A also demonstrated that choriocarcinoma-derived cell lines cluster together, whilst HTR-8/SVneo cells appear in a different branch of the dendogram (Fig. 1A). This was not observed on Card B, but in general the expression of miRNAs detected on this card was low, and therefore, the information may not be sufficient to discriminate between cell lines.

Similarly, by unsupervised hierarchical clustering of the microRNA signatures we identified two major groups of microRNAs, one of which highly expressed in the choriocarcinoma-derived cells and term trophoblast cells, and the other one highly expressed in HTR-8/SVneo cells and expressed by trophoblast cells but almost absent in the choriocarcinoma-derived cells (Fig. 1C and D). A deeper

Table 1
Sequences and chromosome localization of miRNAs with >100 fold-change in HTR-8 cells compared with JEG-3 cells. All of them have been found within the marked unsupervised hierarchically calculated clusters of cards A and B in Fig. 1 and belong to C14MC, C19MC, C19 or the let-7 family.

Up-regulated miRNA (>100-fold) in HTR-8/SVneo versus JEG-3 cells			Down-regulated miRNA (>100-fold) in HTR-8/SVneo versus JEG-3 cells		
miRNA	Sequence (5′–3′)	Locus	miRNA	Sequence (5′–3′)	Locus
miR-127-3p	UCGGAUCCGUCUGAGCUUGGCU	C14MC	miR-371-3p	AAGUGCCGCCAUCUUUUGAGUGU	C19
miR-134	UGUGACUGGUUGACCAGAGGGG	C14MC	miR-372	AAAGUGCUGCGACAUUUGAGCGU	C19
miR-136*	CAUCAUCGUCUCAAAUGAGUCU	C14MC	miR-373	GAAGUGCUUCGAUUUUGGGGUGU	C19
miR-299-5p	UGGUUUACCGUCCCACAUACAU	C14MC	miR-512-3p	AAGUGCUGUCAUAGCUGAGGUC	C19MC
miR-337-5p	GAACGGCUUCAUACAGGAGUU	C14MC	miR-512-5p	CACUCAGCCUUGAGGGCACUUUC	C19MC
miR-369-5p	AGAUCGACCGUGUUAUAUUCGC	C14MC	miR-515-3p	GAGUGCCUUCUUUUGGAGCGUU	C19MC
miR-370	GCCUGCUGGGGUGGAACCUGGU	C14MC	miR-515-5p	UUCUCCAAAAGAAAGCACUUUCUG	C19MC
miR-376a	AUCAUAGAGGAAAAUCCACGU	C14MC	miR-516a-5p	UUCUCGAGGAAAGAAGCACUUUC	C19MC
miR-379	UGGUAGACUAUGGAACGUAGG	C14MC	miR-516b	AUCUGGAGGUAAGAAGCACUUU	C19MC
miR-382	GAAGUUGUUCGUGGUGGAUUCG	C14MC	miR-517a	AUCGUGCAUCCCUUUAGAGUGU	C19MC
miR-409-3p	GAAUGUUGCUCGGUGAACCCCU	C14MC	miR-517*	CCUCUAGAUGGAAGCACUGUCU	C19MC
miR-410	AAUAUAACACAGAUGGCCUGU	C14MC	miR-517b	UCGUGCAUCCCUUUAGAGUGUU	C19MC
miR-411	UAGUAGACCGUAUAGCGUACG	C14MC	miR-517c	AUCGUGCAUCCUUUUAGAGUGU	C19MC
miR-431	UGUCUUGCAGGCCGUCAUGCA	C14MC	miR-518b	CAAAGCGCUCCCCUUUAGAGGU	C19MC
miR-487b	AAUCGUACAGGGUCAUCCACUU	C14MC	miR-518c	CAAAGCGCUUCUCUUUAGAGUGU	C19MC
miR-539	GGAGAAAUUAUCCUUGGUGUGU	C14MC	miR-518c*	UCUCUGGAGGGAAGCACUUUCUG	C19MC
miR-541	UGGUGGGCACAGAAUCUGGACU	C14MC	miR-518d-5p	CUCUAGAGGGAAGCACUUUCUG	C19MC
miR-543	AAACAUUCGCGGUGCACUUCUU	C14MC	miR-518e	AAAGCGCUUCCCUUCAGAGUG	C19MC
miR-654-5p	UGGUGGGCCGCAGAACAUGUGC	C14MC	miR-518e*	CUCUAGAGGGAAGCGCUUUCUG	C19MC
miR-758	UUUGUGACCUGGUCCACUAACC	C14MC	miR-518f	GAAAGCGCUUCUCUUUAGAGG	C19MC
miR-889	UUAAUAUCGGACAACCAUUGU	C14MC	miR-518f*	CUCUAGAGGGAAGCACUUUCUC	C19MC
miR-1247	ACCCGUCCCGUUCGUCCCCGGA	C14	miR-519a	AAAGUGCAUCCUUUUAGAGUGU	C19MC
let-7b	UGAGGUAGUAGGUUGUGUGGUU	C22	miR-519b-3p	AAAGUGCAUCCUUUUAGAGGUU	C19MC
let-7d	AGAGGUAGUAGGUUGCAUAGUU	C9	miR-519c-3p	AAAGUGCAUCUUUUUAGAGGAU	C19MC
let-7e	UGAGGUAGGAGGUUGUAUAGUU	C19	miR-519d	CAAAGUGCCUCCCUUUAGAGUG	C19MC
let-7g	UGAGGUAGUAGUUUGUACAGUU	C3	miR-519e	AAGUGCCUCCUUUUAGAGUGUU	C19MC
let-7c	UGAGGUAGUAGGUUGUAUGGUU	C21	miR-519e*	UUCUCCAAAAGGGAGCACUUUC	C19MC
let-7f	UGAGGUAGUAGAUUGUAUAGUU	C9	miR-520a-3p	AAAGUGCUUCCCUUUGGACUGU	C19MC
let-7i*	CUGCGCAAGCUACUGCCUUGCU	C12	miR-520a-5p	CUCCAGAGGGAAGUACUUUCU	C19MC
			miR-520b	AAAGUGCUUCCUUUUAGAGGG	C19MC
			miR-520c-3p	AAAGUGCUUCCUUUUAGAGGGU	C19MC
			miR-520d-3p	AAAGUGCUUCUCUUUGGUGGGU	C19MC
			miR-520d-5p	CUACAAAGGGAAGCCCUUUC	C19MC
			miR-520e	AAAGUGCUUCCUUUUUGAGGG	C19MC
			miR-520f	AAGUGCUUCCUUUUAGAGGGUU	C19MC
			miR-520g	ACAAAGUGCUUCCCUUUAGAGUGU	C19MC
			miR-520h	ACAAAGUGCUUCCCUUUAGAGU	C19MC
			miR-521	AACGCACUUCCCUUUAGAGUGU	C19MC
			miR-522	AAAAUGGUUCCCUUUAGAGUGU	C19MC
			miR-523	GAACGCGCUUCCCUAUAGAGGGU	C19MC
			miR-524	GAAGGCGCUUCCCUUUGGAGU	C19MC
			miR-524-5p	CUACAAAGGGAAGCACUUUCUC	C19MC
			miR-525-3p	GAAGGCGCUUCCCUUUAGAGCG	C19MC
			miR-525-5p	CUCCAGAGGGAUGCACUUUCU	C19MC
			miR-526b	CUCUUGAGGGAAGCACUUUCUGU	C19MC

analysis revealed that 19 out of the 36 miRNAs belonging to this second group map to chromosome 14 (underlined miRNAs in Fig. 1D). In contrast, 34 out of the 42 miRNAs highly expressed in JEG-3 cells and its hybrids belong to the placenta-specific cluster C19MC, and 3 miRNAs to the miR-371-3 cluster, both encoded in chromosome 19 (Fig. 1C underlined miRNAs). Isolated first and term trophoblast cells express members of all three mentioned miRNA clusters, but at different levels (see below).

For deeper investigation of miRNAs differing between cell lines, those miRNAs with a fold change higher than 100-fold between HTR-8/SVneo cells and JEG-3 cells were further statistically evaluated. Thereof, 22 miRNAs belong to C14MC and are upregulated in HTR-8/SVneo cells while 42 miRNAs belong to C19MC and were downregulated in HTR-8/SVneo cells (Table 1).

Because of their large expression differences between the cell lines, miRNAs within C14MC and C19MC dominate the unsupervised clustering of analyzed samples. The dendogram is mainly organized depending on the statistical power of these principle components. In order to investigate the relevance of further miRNAs, which do not belong to C14MC or C19MC, the unsupervised hierarchical clustering was repeated after depleting their respective results. When only C14MC miRNAs were excluded, the order of the resulting dendogram was the same as the original (Fig. 1E). When C19MC miRNAs were excluded, trophoblast cells appear in a separate branch of the dendogram and closely associated with HTR-8/SVneo cells, which demonstrates that miRNAs belonging to the C19MC are the principle components for the observed similarities between choriocarcinoma-derived cell lines and isolated trophoblast cells (Fig. 1E). The depletion of the combination of both, C19MC and C14MC miRNAs data, did not result in additional changes (Fig. 1E). These results highlight on the one hand the leading relevance of C19MC in distinction of the analyzed cell types, but on the other hand, that the fingerprints and differences between the different analyzed cell types do not depend exclusively on C19MC and C14MC miRNAs.

3.2. MicroRNA of first and third trimester isolated trophoblast cells

MiRNAs exhibiting fold changes higher than 100 between first and third trimester trophoblast cells, were selected for more detailed statistical analysis: 15 miRNAs were upregulated and 31 downregulated in trophoblast cells isolated from term placentae (Table 2). In the group of the downregulated miRNAs, 16 miRNA map on chromosome 14 suggesting a strong decrease of the gene expression of C14MC with gestational age (Table 2). We further focussed on the development of the expression of all miRNAs belonging to C14MC and C19MC from first to third trimester trophoblast cells. A total of 93 miRNAs in both clusters were differentially expressed (Fig. 2A–B). Fourty-six out of 47 miRNAs belonging to C19MC were upregulated (>50%) in third trimester trophoblasts, while 34 of 46 miRNAs belonging to C14MC were downregulated (>50%; Fig. 2).

Table 2
MiRNAs differentially expressed between isolated first and third trimester trophoblast cells. Listed are all miRNAs exhibiting >100-fold change.

miRNAs>100 fold changing in third trimester versus first trimester trophoblast					
Up-regulated			Down-regulated		
miRNA	Fold change	Chr	miRNA	Fold change	Chr
miR-571	30,254.01	4	miR-431	0.0002	14
miR-1178	22,925.07	12	miR-382	0.0004	14
miR-302a	5583.75	4	miR-654-5p	0.0006	14
miR-183#	2773.31	7	miR-23b	0.0008	9
miR-211	1220.31	15	miR-22	0.0011	17
miR-584	1009.38	5	let-7f	0.0015	X
miR-648	771.11	22	miR-505	0.0017	X
miR-663B	361.99	2	miR-7#	0.0023	17
miR-518f#	142.77	19	miR-337-3p	0.0024	14
let-7f-1#	119.21	9	miR-876-5p	0.0028	9
miR-208b	117.06	14	miR-501-5p	0.0033	X
miR-144	115.12	17	miR-376b	0.0037	14
miR-524-5p	114.25	19	miR-409-5p	0.0041	14
miR-25#	102.45	7	miR-889	0.0042	14
			miR-493	0.0043	14
			miR-342-5p	0.0043	14
			miR-363	0.0047	X
			miR-618	0.0048	12
			miR-339-5p	0.005	7
			miR-154	0.0055	14
			miR-15a	0.0057	13
			miR-429	0.006	1
			miR-299-5p	0.006	14
			miR-200a	0.006	1
			miR-656	0.0063	14
			miR-654-3p	0.0074	14
			miR-379	0.0074	14
			miR-369-3p	0.0088	14
			miR-18b	0.0088	X
			miR-331-5p	0.0094	12
			miR-377#	0.01	14

4. Expression of selected miRNAs confirmed by qPCR

For confirmation of array results, we analyzed by qPCR individually the expression of 2 miRNAs representing C14MC (miR-411 and miR-539), 2 miRNAs representing C19MC (miR-519e and miR-518a-5p) and miR-373, a member of the miR-371-3 cluster. In agreement with the arrays, the expression of the miRNAs belonging to C19MC increases from first to third trimester, while the expression of members of C14MC decreases.

As observed in the arrays, HTR-8/SVneo cells differ significantly in the expression of the miRNAs located on the chromosome 19. The levels of miR-518a-5p, miR-519e and miR-373 were 128-, 704.3-, and 79.9-fold higher, respectively, in first trimester trophoblast cells than in HTR-8/SVneo cells, but lower than in all choriocarcinoma-derived cell lines (Fig. 3).

In contrast, the expression of miR-539 in HTR-8/SVneo cells was 30.7-fold higher than in first trimester trophoblast cells, but only slightly different from the choriocarcinoma-derived cells. Notably the expression of miR-411 in 1st trimester trophoblast cells was very high and more similar to that in HTR-8/SVneo rather than to other cell lines. In general, the expression of the mentioned microRNAs was very similar between the JEG-3 cells and its hybrids ACH-3P (JEG-3 derivates fused with 1st trimester trophoblast) and AC1-M59 (JEG-3 derivates fused with 3rd trimester trophoblast). However, a slight increase in the expression of miR-373 (>75%), as well as a decrease in miR-539 (>80%) and miR-411 (>50%) was observable in AC1-M59 cells compared to ACH-3P cells (Fig. 3). These changes are similar to those in trophoblast cells from 1st to 3rd trimester suggesting that the expression of these miRNAs in the fusion-derived cell lines may reflect the respective development stage of the underlying trophoblast cells used for the generation of both cell lines.

Additionally, the expression of 4 further miRNAs which may be related with pregnancy or cancer has been analyzed by qPCR. The results correlate with the array in the up- or down-regulation of the genes but some differences in the fold change values were observed (GEO GSE32346 and Fig. 3).

The expression of all four miRNAs was higher in 3rd than in 1st trimester trophoblast cells, but the differences are little and only in miR-141 a fold-change higher than 2 was observed. The expression of miR-9 was higher in all cell lines compared to primary trophoblast cells while that of miR-141 was significantly lower in all cell lines. The expression of miR-21 and let-7g in JEG-3 and its hybrids was very similar and significantly lower than in HTR-8/SVneo cells.

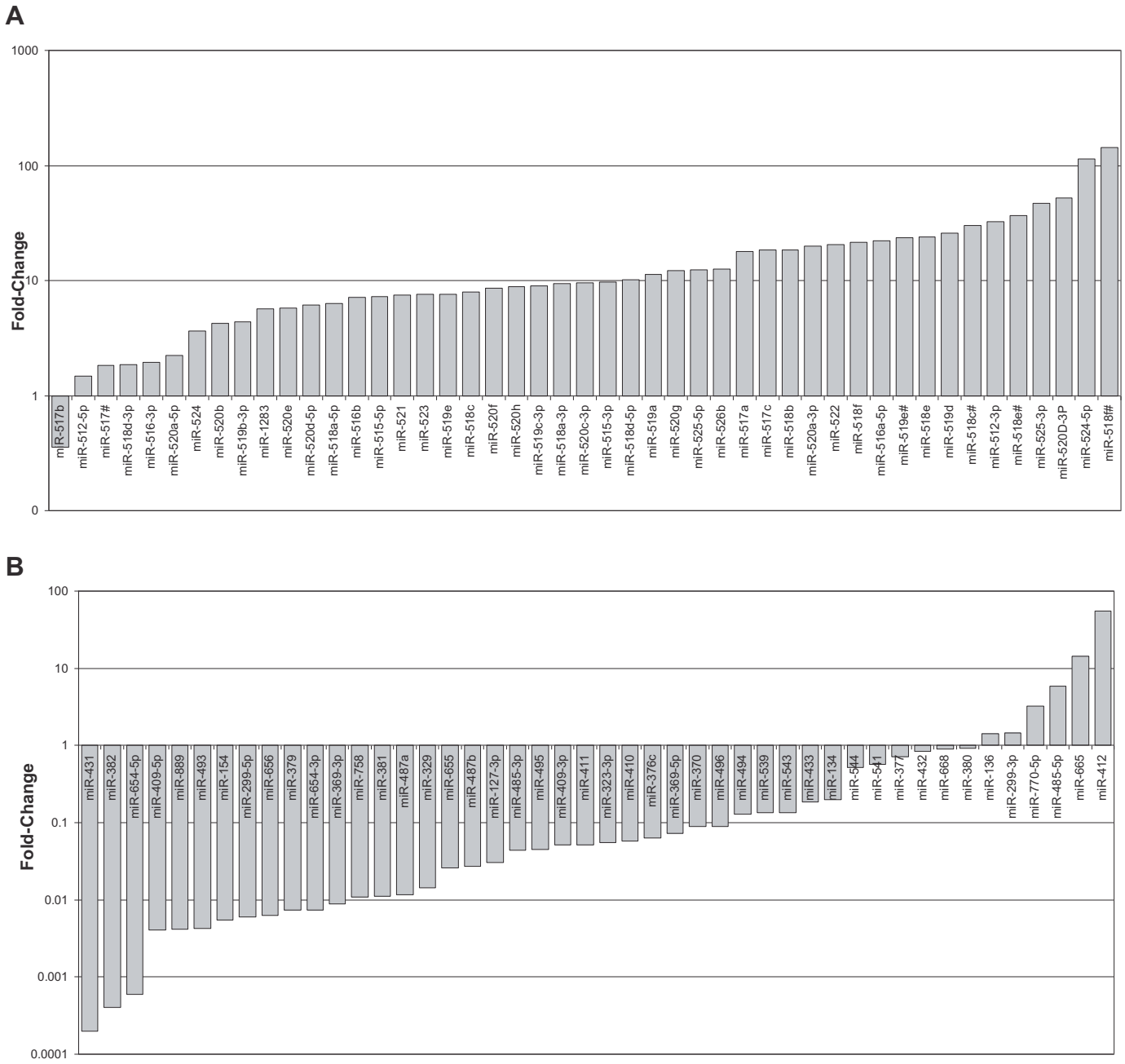

Fig. 2. Relative expression (fold change) of miRNAs belonging to C19MC and C14MC clusters in third trimester compared with first trimester trophoblast cells. Results were extracted from the miRNA arrays (Fig. 1). The horizontal line defines the expression in first trimester trophoblast cells, values above and below this level display gene up- or down-regulation, respectively.

4.1. Expression of miRNAs in first and third trimester trophoblast cells resembles choriocarcinoma cell lines more than HTR-8/SVneo cells

Finally, we compared the expression of those miRNAs with the lowest Ct-values in first and third trimester trophoblast cells with that in cell lines. Twenty miRNAs exhibiting the lowest Ct-values, each in first and third trimester trophoblast cells, were selected for this analysis. This miRNA selection from primary first trimester trophoblast cells map on a variety of chromosomes including 3 miRNAs on chromosome 19, whereas 8 out of the 20 highest expressed miRNA in third trimester trophoblast cells are located on chromosome 19. The heatmap demonstrates that the expression of many of the selected miRNAs have a lower expression in HTR-8/SVneo than the other cell lines (Table 3).

5. Discussion

Recent studies indicate that miRNA expression signatures may be useful for the characterization and prediction of cancer [13], but the investigation of their physiological role in pregnancy and their possible involvement in pregnancy disorders are still incipient. The expression of individual miRNAs as well as miRNA clusters is regulated in a tissue-specific manner. Within its complex organ specific miRNA signature, the placenta expresses the two microRNA clusters C19MC and C14MC. Both are almost placenta-specific, but are also expressed in brain [3,8,19]. Serum levels of some of the C19MC members are altered in preeclampsia [8,20,29]. However, the cellular origin of these miRNAs or their role in the control of trophoblast invasion and other functions are still unknown.

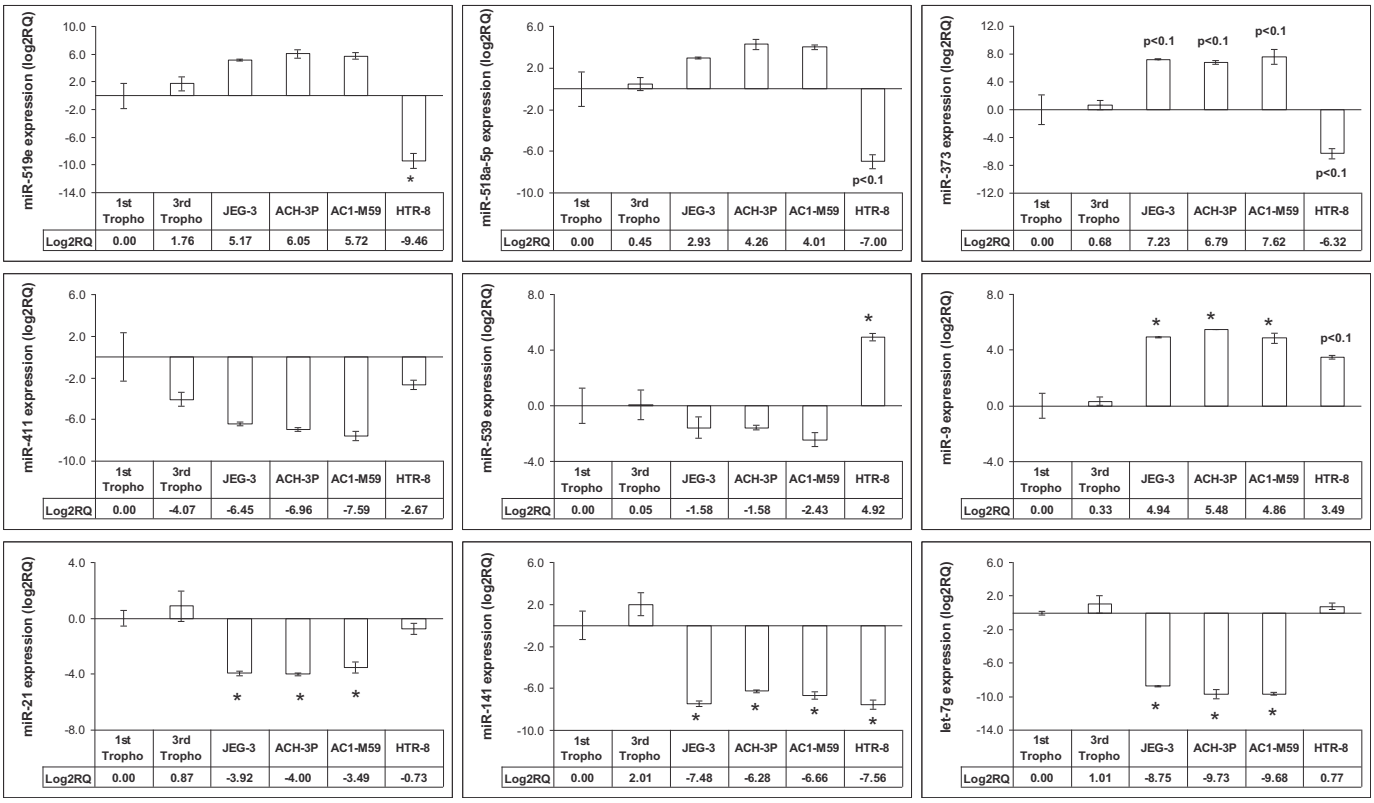

Fig. 3. Confirmation of array data by individual qRT-PCR. Relative expression of miRNAs belonging to either C19MC (miR-519e and miR-518a-5p), miR-371-3 cluster (miR-373) or C14MC (miR-539 and miR-411) as well as further selected miRNAs which have been analyzed in previous studies [26] was analyzed in four cell lines and isolated first and third trimester trophoblast cells. Data is presented as mean fold change (Log2RQ) compared to mean expression in first trimester isolated trophoblast cells ± SE. The fold change has been calculated using the delta-delta-Ct method using first trimester trophoblast cell results as calibrators and RNU44 and RNU48 as endogenous controls. $^{*}p < 0.05$ (when compared with isolated trophoblast cells; one-tailed Student's *t*-test).

A comprehensive study on small RNAs in pregnancy described the miRNA expression profiles of the human first trimester and term placentae. Furthermore, in term placentae whole-villi and terminal-villi have been analyzed separately [18]. We decided to compare these profiles with those of isolated trophoblast cells in the present study. Among the 20 highest expressed miRNAs in first trimester trophoblast cells 5 miRNAs were within the group ($n = 28$ miRNAs) of the highly cloned miRNAs from whole placenta: miR-21, miR-199a, miR-30b, miR-191, miR-24 and miR-30c. Similarly, the comparison of term isolated trophoblast cells and placenta villi revealed seven miRNAs highly expressed in both (miR-517a, miR-200c, miR-30b, miR-24, miR-517c, miR-519a and miR-512-3p). Eight out the 20 miRNAs with the lowest Ct in trophoblast cells in our study belong to C19MC. This is concordant with a very recent study which underlines that the C19MC miRNAs are the most abundant in human term trophoblast [24]. However, it cannot be assumed that exclusively trophoblast cells, but no other cell types within the tissue, express these miRNAs. In our hands, more than 90% of cells were trophoblast cells, but for final clarification it will be necessary to analyze the expression profiles of contaminating cell types separately. The trophoblastic origin of most of the detected miRNAs can also be supported by their detection in the different analyzed trophoblastic cell lines.

For the study of the molecular mechanisms involved in the regulation of trophoblast proliferation and invasion an increasing variety of cell lines are used as models due to the limitations of primary cultures. The investigated cell lines here include the most accepted models: HTR-8/SVneo, JEG-3, AC1-M59 and ACH-3P. However, it is still controversially discussed to which extend they resemble trophoblast cells and how to extrapolate results from these models for generation of hypotheses for the different physiological trophoblast subtypes. On the one hand, HTR-8/SVneo cells have the advantage of being benign first trimester trophoblast cells, but vector transformation as used for their immortalization may induce uncontrolled amplification and splicing of viral DNA resulting in a heterogeneous genotype [25]. On the other hand, choriocarcinoma cells are not virus-treated, but have, due to their malignant origin, different functional characteristics and gene expression patterns from physiological trophoblast cells [30].

Recent studies on several trophoblastic cell lines and isolated primary trophoblast cells demonstrate that mRNAs signatures allow differentiation between choriocarcinoma-derived cell lines, immortalized trophoblast cell lines and primary trophoblast cells [21,31]. Also several functional differences, mainly in regard to invasiveness and proliferation, and in combination with different expression patterns of mRNA and proteins including HLA molecules, have been described between HTR-8/SVneo cells and choriocarcinoma cells [21,32–34]. Similar to these observations, in the current study, we demonstrate that miRNA profiles of the choriocarcinoma-derived cell lines JEG-3, ACH-3P and AC1-M59 share large congruences with each other, but not with HTR-8/SVneo. In comparison with primary first and third trimester trophoblast cells by performing unsupervised hierarchical clustering, miRNA profiles of choriocarcinoma-derived cell lines resemble more the primary trophoblast cells than profiles from HTR-8/SVneo do. These similarities depend mainly on the expression of the miRNA cluster C19MC.

Due to its placenta specificity, it can be expected that alterations of C19MC may be involved in pregnancy pathologies by being their cause or their consequence. In other cells than trophoblast and

Table 3
Heatmapped list and chromosome localization of the 20 (out of 762) miRNAs with lowest Ct-values in isolated first or term trophoblast cells for a rough estimation of cell line dependent distinct expression levels. Mean Ct-values of these miRNAs are listed for all analyzed cell types. Background colors: black: Ct-value < 20; dark gray: Ct-value 20–25; light gray: Ct-value 25–30; white: Ct-value > 30. RNU48: Endogenous control.

MicroRNA	Chromosome	1. trim	3. trim	JEG-3	AC1-M59	ACH-3P	HTR-8
Lowest Ct in 1st trimester							
miR-21	17	17.0	24.3	22.9	21.9	22.1	20.6
miR-24	9, 19	17.0	20.2	21.1	20.3	20.9	19.6
miR-145	5	18.1	23.8	28.8	27.0	28.1	27.7
let-7e	19	18.1	23.1	25.1	27.1	26.0	22.1
miR-19b	13, X	18.2	23.0	17.6	17.8	17.8	20.0
miR-222	X	18.3	23.5	22.4	22.1	21.5	19.8
miR-20a	13	18.8	23.9	19.8	18.8	19.0	22.5
miR-223	X	19.2	24.1	25.6	30.6	30.7	31.6
let-7b	22	19.3	23.3	35.7	40.0	40.0	22.7
miR-125b	11, 21	19.3	24.8	28.5	27.8	28.8	22.3
miR-30b	8	19.4	22.1	20.9	20.4	20.7	21.0
miR-132	17	19.4	26.3	26.8	26.1	26.1	25.3
miR-342-3p	14	19.4	23.0	23.3	23.3	23.7	23.6
miR-199a-3p	19, 1	19.5	25.6	37.6	35.7	35.0	27.8
miR-30c	6, 1	19.6	22.6	20.3	20.0	20.4	21.0
miR-26a	3, 12	19.6	25.2	23.0	21.7	22.1	24.5
miR-191	3	19.7	22.1	21.6	20.8	21.3	21.8
miR-146b-5p	10	19.8	24.9	29.8	27.9	28.6	27.7
miR-106a	X	19.9	23.7	19.2	19.0	19.2	20.9
miR-29a	7	19.9	24.9	26.5	26.2	26.1	23.8
RNU48		17.1	18.9	17.7	17.1	17.6	19.9
Lowest Ct in 3rd trimester							
miR-517a	19	21.0	19.4	18.3	17.7	17.8	37.0
miR-512-3p	19	22.0	19.5	17.0	16.0	16.4	40.0
miR-24	9	17.0	20.2	21.1	20.3	20.9	19.6
miR-517c	19	21.9	20.2	18.5	17.5	17.7	37.0
miR-193b	16	20.0	20.9	19.6	19.2	20.0	23.9
miR-720	3	19.7	21.4	13.8	13.8	19.4	16.4
miR-200c	12	22.4	21.3	27.6	28.4	28.2	29.3
miR-519a	19	22.5	21.5	19.0	17.9	17.9	36.5
miR-519d	19	23.8	21.6	19.5	17.4	18.1	35.5
miR-30b	8	19.4	22.1	20.9	20.4	20.7	21.0
miR-191	3	19.7	22.1	21.6	20.8	21.3	21.8
miR-525-3p	19	25.3	22.2	22.0	21.4	21.8	40.0
miR-518e	19	24.6	22.5	19.3	17.7	17.9	38.5
miR-30c	6	19.6	22.6	20.3	20.0	20.4	21.0
miR-484	16	20.2	22.7	21.0	20.9	21.1	23.1
miR-1290	1	25.3	23.1	15.3	15.5	16.4	19.5
miR-483-5p	11	23.5	22.9	22.7	24.5	24.7	31.8
miR-19b	13, X	18.2	23.0	17.6	17.8	17.8	20.0
miR-342-3p	14	19.4	23.0	23.3	23.3	23.7	23.6
miR-518f	19	24.9	23.0	21.5	19.1	19.7	36.0
RNU48		17.1	18.9	17.7	17.1	17.6	19.9

brain, a distal CpG-rich region on chromosome 19 is hypermethylated, which inhibits the C19MC expression. This region can be demethylated in several human cancers, which leads to expression of the respective miRNAs [16]. It can be argued that C19MC miRNA expression in choriocarcinoma cells derives from their trophoblastic origin, their cancerous properties or from both, which may explain the mostly higher C19MC expression than in primary trophoblast cells. In contrast to C19MC, another placenta (embryonic tissue and brain) specific miRNA cluster, C14MC [29], is highly expressed in HTR-8/SVneo and in primary first and third trimester trophoblast cells, but it is almost absent in the here analyzed choriocarcinoma-derived cell lines. In a previous study, several members of both clusters have been detected in cell-free plasma of pregnant women [17]. The concentrations of members of C19MC increased with the progress of pregnancy [17] and decrease dramatically after delivery [18]. C19MC members can also be detected in exosomes in serum of pregnant women [24]. Our results go in line with these observations. We are providing evidence that the C19MC and C14MC miRNAs are among the most abundant expressed by trophoblast cells but also that their expression correlates with the gestational age: C19MC increases throughout pregnancy while C14MC decreases. The expression levels of C19MC in the two hybrid cell lines ACH-3P and AC1-M59, which were developed by fusion of JEG-3 with isolated trophoblast cells from first and third trimester placentae, seems to reflect the gestational age of the fused trophoblast cells, as an increase of miR-371-3 cluster and a decrease of C14MC expression from ACH-3P to AC1-M59 was detectable.

A further major difference between choriocarcinoma and HTR-8/SVneo cells is, that JEG-3 cells and their hybrids express the human embryonic stem cell specific miR-371-3 cluster, while HTR-8/SVneo do not. This miRNA cluster was also observed in primary trophoblast cells and its expression increases slightly in third trimester. The miR-371-3 cluster is thought to be involved in stem cell maintenance [35] and cell differentiation [36]. Therefore, it is expectable that it also plays a functional role in the trophoblast differentiation.

We further analyzed the expression of two pregnancy-associated miRNAs by qPCR: miR-21 and miR-141. As discussed above, in another study, miR-21 was the highest cloned miRNA from human placenta [18]. In concordance, we also found it highly expressed in all tested cells (Ct < 23). Indeed, it showed the lowest Ct-value in first trimester isolated trophoblast cells. MiR-21 is also strongly secreted by human mesenchymal stem cells derived from embryonic stem cells [37], and its overexpression in a variety of cancer types [38] which correlates with their elevated proliferation and

invasiveness [39,40]. Based on the high expression in trophoblast cells, it may be argued that miR-21 is involved in enhanced trophoblast proliferation and invasion during the initial steps of implantation and placentation. Similarly to miR-21, miR-141 was highly expressed in all tested cells and approximately 128fold higher in first trimester trophoblast cells. Its downregulation resulted in a decrease of trophoblast proliferation [26]. Both, miR-21 and miR-141, can be found in significant amounts in the maternal circulation. They are delivered in an exosome-mediated manner [17,18]. Therefore, their expression level may be useful for monitoring regular pregnancy progress, since it is to expect that they mirror the status of the placenta.

Finally, the expression of two exemplary miRNAs associated with cancer was measured. MiR-9 expression correlates with tumor-grade and metastatic status in breast and cervical cancer [39,41]. The expression of miR-9 in all trophoblastic cell lines was similar and higher than in isolated trophoblast cells. This may be a result of the transformation with SV40 in HTR-8/SVneo or reflect their choriocarcinoma origin. On the other hand, low expression of let-7g in the choriocarcinoma cells, but not in the HTR-8/SVneo, goes in line with the observation that suggests let-7g as a tumor suppressor. It has previously been reported that low expression of let-7g is associated with unfavorable outcome in gastric cancer [42] and that let-7g inhibits proliferation of hepatocellular carcinoma [43]. Although the expression pattern of these two miRNA in malignant and non-malignant trophoblastic cells corresponds to that of other cell types and their malignant counterpart, the observation cannot be generalized for other miRNA associated with cancer.

However, the qPCR results of miR-9 and let-7g expression confirm the observations of the array data. In general, the qPCR data of the 9 individually analyzed miRNA supported widely the array results, although a few quantitative differences appeared.

6. Conclusion

Our study provides a comprehensive encyclopedia of the microRNA expression profile of four cell lines widely used as models of trophoblast cells, and their comparison with primary isolated trophoblast cells from first and third trimester. In regard to the current international discussion about the nature of HTR-8/SVneo cells, this study confirms their close relationship with primary trophoblast cells, but it also exhibits large inequalities. Simultaneously, it demonstrates that also choriocarcinoma cell lines express trophoblast-specific miRNA patterns, but it displays also numerous dissimilarities. Therefore, it may be concluded that the use of a single cell line as a model for trophoblast cells carries the risk of misleading to erroneous hypotheses, Results obtained from such models require validation through the analysis of primary cells.

The here presented encyclopedia may be useful for comparison of trophoblastic cells with other cell types and tissues, for interpretation of any experimental results from the analyzed cell lines, for future analyses of function of major trophoblast-related miRNA clusters, or for selection of novel miRNA targets for further investigations.

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Pregnancy-associated miRNA-clusters

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ABSTRACT

MicroRNAs (miRNAs) are expressed in the placenta and can be detected in maternal plasma. An increasing number of studies have been published on the cellular origin, distribution and function of miRNAs in pregnancy. Specific miRNA profiles have been described for the placenta, maternal plasma and several pregnancy disorders. It has been observed that numerous miRNAs, which are predominantly or exclusively expressed during pregnancy, are clustered in chromosomal regions, may be controlled by the same promoters, may have similar seed regions and targets, and work synergistically. The three most eminent clusters are the chromosome 19 miRNA cluster (C19MC), C14MC and miR-371-3 cluster, which is also localized on chromosome 19. MiRNA members of these clusters are not only detected in the placenta, but also in other compartments, e.g. in serum where they have the potential to become novel biomarkers of pregnancy disorders. Additionally, some members are also expressed in a variety of tumors. Antagonism of selected miRNAs or their targets may lead to novel strategies for the development of new drug classes in pregnancy disorders or other diseases. This review summarizes current knowledge on the pregnancy-related miRNA clusters – the C19MC, C14MC and miR-371-3 cluster – in regard to pregnancy and also other, mostly pathological circumstances.

1. Introduction

The study of gene regulation beyond the DNA transcription has provided important novel interpretations of genetic research and has generated great interest in the field of human reproduction. Epigenetics is defined as the study of changes in gene expression that are not caused by changes in the DNA sequence (Maccani and Marsit, 2009; Wilkins-Haug, 2009). Four main mechanisms are considered as epigenetic processes: DNA methylation, imprinting, histone modification, and small RNA-mediated control, specifically through microRNAs (miRNAs) (Maccani and Marsit, 2009).

MicroRNAs are single-stranded RNA molecules which act as translational repressors by either degrading or inhibiting translation of mRNA targets (reviewed by Morales Prieto and Markert, 2011). miRNAs control numerous cellular processes including metabolism, cell proliferation, apoptosis, and differentiation in almost all cell types (Seitz et al., 2004; Cheng et al., 2005; Bueno et al., 2008; Chen et al., 2010). MiRNAs are known to be involved in processes associated with establishment and maintenance of pregnancy including preparation of the endometrium for implantation (Pan and Chegini, 2008), control of genes associated with inflammatory responses (Chakrabarty et al., 2007), and regulation of immune tolerance-associated genes, such as HLA-G (Veit and Chies, 2009).

During embryogenesis, critical periods are controlled by epigenetic modification of genes: gamete development, preimplantation embryo development, and placentation (Wilkins-Haug, 2009). Recent studies have demonstrated a

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placenta-specific miRNA profile (Liang et al., 2007), which seems to be dominated by miRNAs expressed in trophoblast cells (Donker et al., 2012; Morales-Prieto et al., 2012). This expression is, at least partly, reflected in the maternal plasma (Chim et al., 2008; Luo et al., 2009) and includes the expression of numerous miRNAs localized within three miRNA clusters: chromosome 14 miRNA cluster (C14MC), C19MC and the miR-371-3 cluster (Liang et al., 2007; Miura et al., 2010; Morales-Prieto et al., 2012). Expression of miRNAs from these clusters changes throughout pregnancy (Miura et al., 2010; Morales-Prieto et al., 2012) and differs among placentas from patients with preterm labor compared to normal term pregnancies (Mayor-Lynn et al., 2011). Remarkably, these clusters are located within imprinted genes which are known to be involved in human embryonic development and to play important roles in the regulation of cellular differentiation and fate (Tsai et al., 2009).

In this review, we summarize the current information about the expression of the pregnancy-related miRNA clusters – C14MC, C19MC and miR-371-3 cluster – their correlation with pregnancy disorders and their possible relevance as biomarkers for the detection and treatment of pregnancy-associated diseases.

2. The chromosome 14 microRNA cluster (C14MC)

C14MC has also been referred to as the *Mirg* cluster (Bortolin-Cavaille et al., 2009), the miR-379/miR-410 cluster (Noguer-Dance et al., 2010) or the miR-379/miR-656 cluster (Glazov et al., 2008). It is the largest described miRNA cluster and comprises 52 miRNA genes located within two closely neighboring segments spanning about 40 kb (Fig. 1) (Seitz et al., 2004; Gardiner et al., 2012; Morales-Prieto et al., 2012). Located at the imprinted DLK-DIO3 domain on the human 14q32 chromosomal interval (mouse distal chromosome 12), C14MC is exclusively expressed from the maternally inherited chromosome and is regulated by methylation of a distal Intergenic Germline-derived Differentially Methylated Region (IG-DMR) (Fig. 1) (Seitz et al., 2004). The expression pattern of this cluster revealed a tissue-specific expression in mice: C14MC is mostly expressed in developing embryonic (head and trunk) and placental tissues, and in adults, it is restricted to brain (Seitz et al., 2004). Similarly, the analysis of healthy human tissues revealed that some members of C14MC are predominantly expressed in placenta or epithelial tissues (Liang et al., 2007).

The origin of C14MC dates back to a precursor sequence which appeared approximately 100 million years ago in an early mammalian ancestor after the radiation of Metatheria (marsupial) or Prototheria (monotreme) (Glazov et al., 2008). This sequence was amplified by tandem duplication resulting in a ~45 kb genomic region that encompasses the C14MC region (Hertel et al., 2006; Glazov et al., 2008). The cluster has been preserved without significant structural changes and is uniquely found in eutherian species (the placental mammals) (Glazov et al., 2008; Bortolin-Cavaille et al., 2009). This suggests that C14MC miRNAs may play important biological roles in this animal lineage, including the control of neurogenesis, embryonic development, transcriptional regulation, and RNA metabolism (Grun et al., 2005). Therefore, it has been suggested that evolution of the placental mammals was facilitated by this miRNA cluster (Glazov et al., 2008).

3. The chromosome 19 microRNA cluster (C19MC)

C19MC is one of the largest miRNA gene clusters in human, maps to chromosome 19q13.41, and spans a ~100 kb long region. C19MC miRNAs are primate-specific, conserved in humans and comprise 46 miRNA genes (Fig. 2) (Bentwich et al., 2005; Bortolin-Cavaille et al., 2009; Lin et al., 2010). The C19MC members share common seed sequences and are supposed to be originated from a common ancestor, which might be a member of the miR-371-3 cluster (Zhang et al., 2008).

Similar to C14MC, C19MC is located within imprinted genes and is only expressed from the paternally inherited chromosome. Its expression is controlled by methylation at the upstream CpG rich promoter region at 17.6 kb of C19MC (Noguer-Dance et al., 2010). Remarkably, C19MC miRNA genes are enriched by dispersed Alu elements approximately 10 times more than other regions, suggesting a co-evolution of Alu sequences and miRNAs (Zhang et al., 2008; Lehnert et al., 2009). The functions of these repeat elements and their cooperation with miRNAs remain unclear. Nevertheless, some reports suggest an involvement of these sequences in the regulation of the genome structure and gene expression (Zhang et al., 2008; Lehnert et al., 2009). Moreover, the expression of miRNAs seems to be partly regulated by Alu elements (Gu et al., 2009; Lehnert et al., 2009).

The expression of C19MC is mainly restricted to the reproductive system and placenta (Bentwich et al., 2005; Liang et al., 2007; Lin et al., 2010), although expression of miR-498 has been reported in fetal brain (20 weeks) (Flor and Bullerdiek, 2012), and some C19MC members are highly expressed in human embryonic stem cells (ESCs) (Bar et al., 2008; Ren et al., 2009). In human and chimpanzee ESCs, the number of miRNA genes which map to the chromosome 19 is strongly increased (Cao et al., 2008). Furthermore, no homologues of this miRNA cluster have been found in rat, mouse or dog which emphasizes its primate-specificity (Zhang et al., 2008). Based on these observations, it has been hypothesized that C19MC may be related with human primate evolution and important for embryo development (Tsai et al., 2009).

4. The miR-371-3 cluster

The miR-371-3 cluster consists mainly of 3 miRNAs sharing the same seed sequence "AAG UGC": hsa-miR-371a-3p, hsa-miR-372 and hsa-miR-373-3p. Two miRNAs synthesized from the opposite site of the pre-microRNA (hsa-miR-371-5p and hsa-miR-373-5p), as well as hsa-miR-371b-3p complete the cluster (Griffiths-Jones et al., 2006; Persson et al., 2011). The miR-371-3 cluster in humans is part of a superfamily of miRNAs sharing the same seed sequence, which also includes the mouse homologue cluster miR-290-295 (Houbaviy et al., 2003).

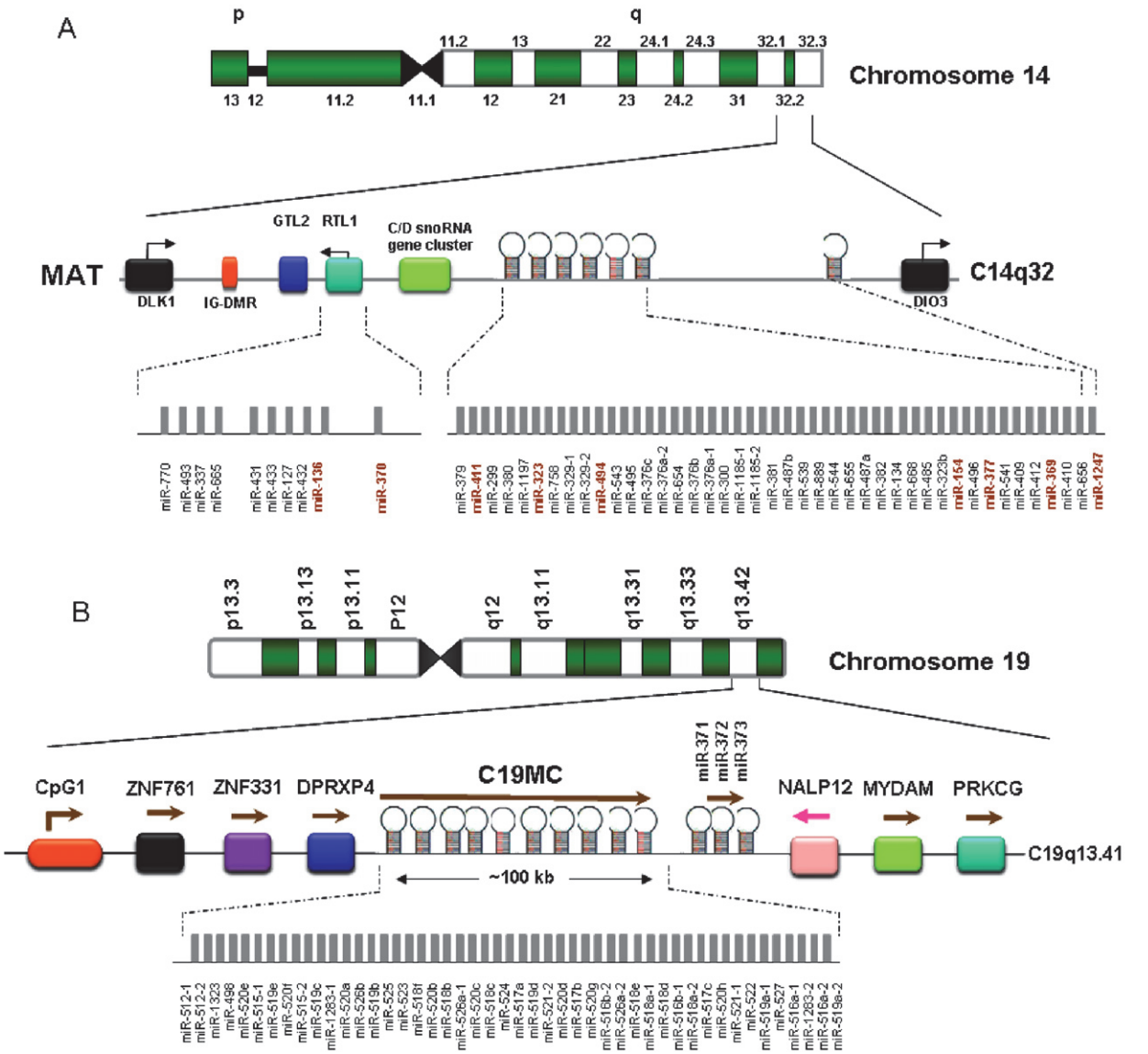

Fig. 1. Schematic representation and genomic organization of chromosome 14 and 19 containing miRNA clusters. (A) Chromosome 14 miRNA cluster (C14MC) in the imprinted DLK-DIO3 domain on the human 14q32 chromosomal interval. Highlighted miRNAs are relevant in pregnancy (*adapted after* Cairo et al., 2010). (B) C19MC (Hg19: 54 169 933–54 265 683) and miR-371-3 cluster mapping at chromosome 19q13.42 (*modified from* Noguer-Dance et al., 2010).

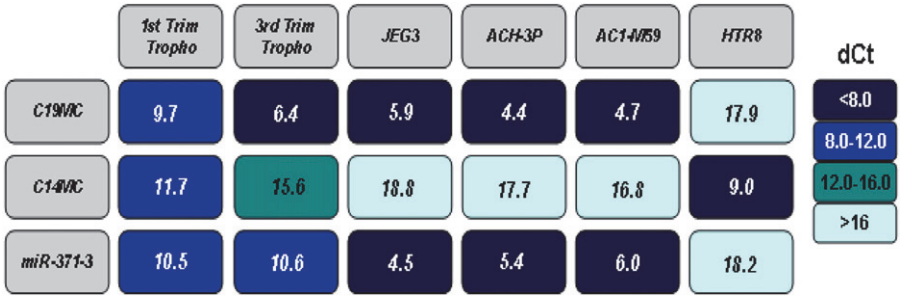

Fig. 2. Heat mapped expression of pregnancy-related microRNA clusters in trophoblast cells. Data is presented as mean of dCt of all miRNAs in each cluster. High expression is represented by dark blue (adapted after Morales-Prieto et al., 2012).

The miR-371-3 cluster is located on chromosome 19 within a 1050 bp region adjacent to the C19MC cluster (Fig. 2) (Suh et al., 2004). Similar to C14MC and C19MC, this cluster is conserved in mammals only (Houbaviy et al., 2003) and is predominantly expressed in the placenta (Bentwich et al., 2005). Interestingly, the members of the miR-371-3 cluster are highly expressed in human ESCs (Laurent et al., 2008) and their levels decrease during development (Suh et al., 2004), possibly marking a "naïve" state of ESCs with the potential to enhance reprogramming of fibroblasts to induced pluripotent stem cells (Wilson et al., 2009; Kim et al., 2011; Subramanyam et al., 2011). Embryonic stem cell-specific cell cycle regulating (ESCC-)miRNAs such as the murine miR-290 cluster or the miR-371-3 cluster are essential for cell cycle maintenance (Wang et al., 2008; Qi et al., 2009) and for regulation of proliferation and apoptosis (Voorhoeve et al., 2006; Cho et al., 2009).

5. Circulating placenta-derived microRNA as novel biomarkers

MicroRNA profiling of human placenta tissues revealed high expression of the clusters C19MC, C14MC and miR-371-3. Interestingly, their expression changes with the gestational age and even differences between whole and terminal villi can be observed (Luo et al., 2009). Recent reports have demonstrated that the placenta miRNome comprises mostly miRNAs belonging to the C14MC and C19MC in the first and third trimester, respectively (Liang et al., 2007; Morales-Prieto et al., 2012). Further, *in situ* hybridization of some C19MC members revealed that the main source of these miRNAs is the trophoblast layer with dominant signals in the syncytiotrophoblast (Zhang et al., 2008; Luo et al., 2009).

The study of the trophoblast cell miRNome revealed that C14MC members are among the highest miRNAs expressed in primary first trimester trophoblast cells and also in the immortalized cell line HTR-8/SVneo (Morales-Prieto et al., 2012). In contrast, miRNA expression of isolated third trimester trophoblast cells was more similar to that of choriocarcinoma cell lines (JEG-3 and derivates) with a predominance of C19MC and the miR-371-3 cluster (Donker et al., 2012; Morales-Prieto et al., 2012). Similar to the observations in maternal plasma, levels of C14MC and C19MC change throughout pregnancy. C14MC is highly expressed by first trimester trophoblast cells but decreases in third trimester. Conversely, C19MC is low expressed during first trimester but highly expressed at the end of pregnancy (Morales Prieto and Markert, 2011) (Fig. 2). Nevertheless, the expression of these clusters may not be exclusive of trophoblast cells. A recent report demonstrated expression of C19MC members in mesenchymal stem cells originated from the villous mesenchymal core, which was free of trophoblast cells (Flor et al., 2012).

Recently, it has been demonstrated that trophoblast and stromal cells of the villi release exosomes into the maternal circulation, which may contain miRNAs belonging to the C19MC (Luo et al., 2009). One of the most interesting features of the circulating miRNAs is their high stability which enables them to resist enzymatic degradation, freeze-thaw cycles and extreme pH conditions (Mitchell et al., 2008; Zhao et al., 2012). Due to these characteristics miRNAs have been proposed as potential blood-based biomarkers for the detection of tumors (Cortez and Calin, 2009). Similarly, circulating miRNA may serve as biomarkers for the detection of pregnancy-associated diseases (Donker et al., 2012; Hromadnikova et al., 2012).

Expression of miRNAs in pregnant women differs from that of non-pregnant women and thus, pregnancy-related miRNAs may become potential markers for the establishment of pregnancy (Reid et al., 2010). Some pregnancy-associated miRNAs, such as miR-526a and miR-527 are 600-fold higher in serum of third trimester pregnant women than in non-pregnant women and allow, as also miR-520d-5p, discrimination between both groups (Gilad et al., 2008). After termination of pregnancy, plasma levels of pregnancy-associated miRNAs belonging to both C14MC and C19MC decrease significantly, which underlines their placental origin (Miura et al., 2010; Kotlabova et al., 2011). Expression of C19MC has been investigated in order to identify miRNAs with diagnostic potential. The findings revealed high expression of these miRNAs in placenta tissue, trophoblastic cells and maternal plasma (Table 1).

The abundant expression of pregnancy-related miRNA clusters in normal placentas also suggests that their alteration may be related with pregnancy-associated diseases such as preeclampsia or growth retardation. Only few studies have been published in this field and there is little overlap among the data on C19MC and C14MC members. For instance, miR-154* (in C14MC) was found initially up-regulated in placenta tissue of patients with preeclampsia associated with small-for-gestational-age (Pineles et al., 2007), but a second study found it down-regulated in severe preeclamptic placentas (Zhu et al., 2009). Besides miR-154*, other C14MC species were found down-regulated in preeclamptic placentas: miR-1247 (Enquobahrie et al., 2011), miR-411 and -377 (Zhu et al., 2009). In contrast, some members of the C19MC were found up-regulated: miR-517*, -518b and -519* (Zhu et al., 2009), and miR-520a-3p, -518f, -517c, -518c, -525-5p, -526b and -519e (Ishibashi et al., 2012). In addition, another comprehensive analysis of 820 miRNAs showed no differences in their expression among normal placentas and those from patients with preeclampsia, but reported changes in placentas of preterm labor when compared with the normal counterparts: Overexpression of members of C19MC (miR-517a, -518b, -512-3p and -526b) and C14MC (miR-431, -539 and -495) (Mayor-Lynn et al., 2011). Remarkably, none of the above mentioned miRNAs was simultaneously found in two different studies. In fact, among the publications summarized in this review, only miR-210 was found commonly up-regulated in all studies suggesting its potential as biomarker for preeclampsia. Further analysis revealed that miR-210 but also miR-518c target hydroxysteroid (17-β) dehydrogenase 1 (HSD17B1), a steroidogenetic enzyme which is decreased in preeclamptic placentas and has been considered a potential prognostic factor for preeclampsia (Ishibashi et al., 2012).

Nonetheless, the use of miRNA species as biomarkers for preeclampsia is still controversial. A recent study reported

Table 1
Expression of C19MC miRNAs in placenta tissue, isolated trophoblast cells from first/third trimester placentas and maternal plasma.

miRNA	Expression	References	miRNA	Expression	References
miR-498	P, T, MP	Bentwich et al. (2005), Liang et al. (2007), Bortolin-Cavaille et al. (2009), Miura et al. (2010), Donker et al. (2012)	miR-519b-3p	F, T	Donker et al. (2012), Morales-Prieto et al. (2012)
miR-512-1	P	Bortolin-Cavaille et al. (2009)	miR-519c	P, F	Bentwich et al. (2005), Bortolin-Cavaille et al. (2009), Morales-Prieto et al. (2012)
miR-512-2	P	Bortolin-Cavaille et al. (2009)	miR-519c-3p	F, T	Donker et al. (2012), Morales-Prieto et al. (2012)
miR-512-3p	F, T, MP,	Gilad et al. (2008), Luo et al. (2009), Donker et al. (2012), Morales-Prieto et al. (2012)	miR-519d	P, F, T, MP	Bentwich et al. (2005), Gilad et al. (2008), Bortolin-Cavaille et al. (2009), Miura et al. (2010), Kotlabova et al. (2011), Donker et al. (2012), Morales-Prieto et al. (2012)
miR-512-5p	F, T, MP	Kotlabova et al. (2011), Donker et al. (2012), Morales-Prieto et al. (2012)	miR-519e	P, F, T	Bentwich et al. (2005), Liang et al. (2007), Bortolin-Cavaille et al. (2009), Morales-Prieto et al. (2012)
miR-515-1	P	Bortolin-Cavaille et al. (2009)	miR-519e#	F, T, MP	Gilad et al. (2008), Morales-Prieto et al. (2012)
miR-515-2	P	Bortolin-Cavaille et al. (2009)	miR-520a	P	Bentwich et al. (2005), Liang et al. (2007), Bortolin-Cavaille et al. (2009)
miR-515-3p	P, F, T, MP	Bentwich et al. (2005), Gilad et al. (2008), Miura et al. (2010), Kotlabova et al. (2011), Donker et al. (2012), Morales-Prieto et al. (2012)	miR-520a*	MP	Kotlabova et al. (2011)
miR-515-5p	P, F, T, MP,	Liang et al. (2007), Morales-Prieto et al. (2012)	miR-520a-3p	F, T	Morales-Prieto et al. (2012)
miR-516-1	P	Liang et al. (2007), Bortolin-Cavaille et al. (2009)	miR-520a-5p	F, T, MP	Gilad et al. (2008), Donker et al. (2012), Morales-Prieto et al. (2012)
miR-516-2	P	Liang et al. (2007)	miR-520b	P, F, T	Bentwich et al. (2005), Liang et al. (2007), Bortolin-Cavaille et al. (2009), Morales-Prieto et al. (2012)
miR-516-3	P	Liang et al. (2007), Bortolin-Cavaille et al. (2009)	miR-520c	P	Bentwich et al. (2005), Liang et al. (2007), Bortolin-Cavaille et al. (2009)
miR-516-4	P	Bentwich et al. (2005), Morales-Prieto et al. (2012)	miR-520c-3p	F, T	Donker et al. (2012), Morales-Prieto et al. (2012)
miR-516-3p	P, F, T	Bentwich et al. (2005), Kotlabova et al. (2011), Morales-Prieto et al. (2012)	miR-520d	P	Bentwich et al. (2005), Bortolin-Cavaille et al. (2009)
miR-516-5p	P, MP	Luo et al. (2009), Morales-Prieto et al. (2012)	miR-520d-3p	F, T	Morales-Prieto et al. (2012)
miR-516a-5p	F, T	Luo et al. (2009), Morales-Prieto et al. (2012)	miR-520d-5p	F, T, MP	Gilad et al. (2008), Augello et al. (2012), Morales-Prieto et al. (2012)
miR-516b	F, T	Liang et al. (2007), Morales-Prieto et al. (2012)	miR-520e	P, F, T	Bentwich et al. (2005), Liang et al. (2007), Bortolin-Cavaille et al. (2009), Morales-Prieto et al. (2012)
miR-516b2-3	P	Bentwich et al. (2005), Gilad et al. (2008), Miura et al. (2010), Kotlabova et al. (2011), Donker et al. (2012), Morales-Prieto et al. (2012)	miR-520f	P, F, T	Bentwich et al. (2005), Liang et al. (2007), Bortolin-Cavaille et al. (2009), Morales-Prieto et al. (2012)
miR-517#	F, T, MP	Gilad et al. (2008), Kotlabova et al. (2011), Morales-Prieto et al. (2012)	miR-520g	P, F, T	Bentwich et al. (2005), Donker et al. (2012), Morales-Prieto et al. (2012)
miR-517a	P, F, T, MP	Bentwich et al. (2005), Liang et al. (2007), Bortolin-Cavaille et al. (2009), Luo et al. (2009), Miura et al. (2010), Donker et al. (2012), Morales-Prieto et al. (2012)	miR-520h	P, F, T, MP	Bentwich et al. (2005), Kotlabova et al. (2011), Donker et al. (2012), Morales-Prieto et al. (2012)

Table 1 (*Continued*)

miRNA	Expression	References	miRNA	Expression	References
miR-517b	P, F, T,	Bentwich et al. (2005), Liang et al. (2007), Bortolin-Cavaille et al. (2009), Luo et al. (2009), Donker et al. (2012)	miR-521	P, F, T, MP	Bentwich et al. (2005), Gilad et al. (2008), Donker et al. (2012), Morales-Prieto et al. (2012)
miR-517c	P, F, T, MP	Bentwich et al. (2005), Liang et al. (2007), Bortolin-Cavaille et al. (2009), Luo et al. (2009), Miura et al. (2010), Donker et al. (2012), Morales-Prieto et al. (2012)	miR-521-1	P	Liang et al. (2007)
miR-517c*	MP	Miura et al. (2010)	miR-521-2	P	Liang et al. (2007), Bortolin-Cavaille et al. (2009)
miR-518a	P	Bentwich et al. (2005), Luo et al. (2009)	miR-522	P, F, T	Bentwich et al. (2005), Bortolin-Cavaille et al. (2009), Donker et al. (2012), Morales-Prieto et al. (2012)
miR-518a-1	P	Liang et al. (2007), Bortolin-Cavaille et al. (2009)	miR-523	P, F, T, MP	Bentwich et al. (2005), Liang et al. (2007), Gilad et al. (2008), Bortolin-Cavaille et al. (2009), Morales-Prieto et al. (2012)
miR-518a-3p	P, F, T, MP	Liang et al. (2007), Gilad et al. (2008), Donker et al. (2012), Morales-Prieto et al. (2012)	miR-524	P, F, T, MP	Bentwich et al. (2005), Liang et al. (2007), Gilad et al. (2008), Bortolin-Cavaille et al. (2009), Morales-Prieto et al. (2012)
miR-518a-5p	F, T	Morales-Prieto et al. (2012)	miR-524*	MP	Gilad et al. (2008)
miR-518b	P, F, T, MP	Bentwich et al. (2005), Bortolin-Cavaille et al. (2009), Luo et al. (2009), Miura et al. (2010), Kotlabova et al. (2011), Donker et al. (2012), Morales-Prieto et al. (2012)	miR-524-3p	T	Donker et al. (2012)
miR-518c	P, F, T, MP	Bentwich et al. (2005), Liang et al. (2007), Bortolin-Cavaille et al. (2009), Miura et al. (2010), Donker et al. (2012), Morales-Prieto et al. (2012)	miR-524-5p	F, T, MP	Kotlabova et al. (2011), Morales-Prieto et al. (2012)
miR-518c#	F, T	Morales-Prieto et al. (2012)	miR-525	P	Bentwich et al. (2005), Gilad et al. (2008), Bortolin-Cavaille et al. (2009), Kotlabova et al. (2011)
miR-518d	P, MP	Bentwich et al. (2005), Liang et al. (2007), Gilad et al. (2008), Bortolin-Cavaille et al. (2009)	miR-525-3p	F, T, MP	Miura et al. (2010), Morales-Prieto et al. (2012)
miR-518d-3p	F, T	Morales-Prieto et al. (2012)	miR-525-5p	F, T, MP	Kotlabova et al. (2011), Donker et al. (2012), Morales-Prieto et al. (2012)
miR-518d-5p	F, T	Morales-Prieto et al. (2012)	miR-526a	P, MP	Gilad et al. (2008), Miura et al. (2010), Kotlabova et al. (2011)
miR-518e	P, F, T, MP	Gilad et al. (2008), Bortolin-Cavaille et al. (2009), Luo et al. (2009), Donker et al. (2012), Morales-Prieto et al. (2012)	miR-526a-1	P	Liang et al. (2007), Bortolin-Cavaille et al. (2009)
miR-518e#	F, T	Morales-Prieto et al. (2012)	miR-526a-2	P	Liang et al. (2007)
miR-518f	P, F, T	Bortolin-Cavaille et al. (2009), Luo et al. (2009), Donker et al. (2012), Morales-Prieto et al. (2012)	miR-526b	P, F, T, MP	Bentwich et al. (2005), Liang et al. (2007), Miura et al. (2010), Kotlabova et al. (2011), Donker et al. (2012), Morales-Prieto et al. (2012)
miR-518f#	F, T	Morales-Prieto et al. (2012)	miR-526c	P, MP	Bentwich et al. (2005), Liang et al. (2007), Gilad et al. (2008)
miR-519a	P, F, T, MP	Bentwich et al. (2005), Luo et al. (2009), Miura et al. (2010), Kotlabova et al. (2011), Morales-Prieto et al. (2012)	miR-527	P, MP	Bentwich et al. (2005), Liang et al. (2007), Gilad et al. (2008)
miR-519a*	MP	Kotlabova et al. (2011)	miR-1283	F, T	Morales-Prieto et al. (2012)
miR-519a-1	P	Bortolin-Cavaille et al. (2009)	miR-1283-1	P	Liang et al. (2007)
miR-519a-2	P	Bortolin-Cavaille et al. (2009)	miR-1283-2	P	Liang et al. (2007)
miR-519b	P	Bentwich et al. (2005), Liang et al. (2007), Bortolin-Cavaille et al. (2009), Morales-Prieto et al. (2012)	miR-1323	P, T	Bortolin-Cavaille et al. (2009), Donker et al. (2012)

P, placenta tissues; F, trophoblast cells isolated from first trimester placentas; T, trophoblast cells isolated from normal term placentas; MP, maternal plasma.

Table 2
C14MC members with published target or associated with pregnancy pathologies.

miRNA	Target	Expression	Associated pathologies	References
hsa-miR-136			Preterm labor Gliomas	Lavon et al. (2010), Mouillet et al. (2011)
hsa-miR-154			Preterm labor Neuroblastoma Gliomas	Chim et al. (2008), Zhu et al. (2009), Lavon et al. (2010), Miura et al. (2010), Enquobahrie et al. (2011), Gattolliat et al. (2011), Mouillet et al. (2011)
hsa-miR-154*		P, T, MP	↑↓**PE** Gliomas Neuroblastoma	Pineles et al. (2007), Chim et al. (2008), Zhu et al. (2009), Lavon et al. (2010), Miura et al. (2010), Gattolliat et al. (2011), Mouillet et al. (2011)
hsa-miR-299	OPN	MP	Neuroblastoma Gliomas	Chim et al. (2008), Lavon et al. (2010), Gattolliat et al. (2011)
hsa-miR-323		P, F, T, MP	**Ectopic pregnancy** Neuroblastoma Medulloblastoma Gliomas Medullary thyroid carcinoma	Chim et al. (2008), Lavon et al. (2010), Gattolliat et al. (2011)
hsa-miR-329	PDGFA, E2FI		Neuroblastoma	Lavon et al. (2010), Miura et al. (2010), Gattolliat et al. (2011), Genovesi et al. (2011), Zhao et al. (2012)
hsa-miR-342	EVL, PDGFRA	MP	↑**sPE** Multiple myeloma	Haller et al. (2010), Wu et al. (2012)
hsa-miR-369			Preterm labor Gliomas	Haller et al. (2010), Gattolliat et al. (2011)
hsa-miR-370	KITLG, NFI		Preterm labor Acute myeloic leukemia Gliomas Gastrointestinal stromal tumors	Lavon et al. (2010), Mouillet et al. (2011)
hsa-miR-376	CDKNIA		Medulloblastoma	Haller et al. (2010), Lavon et al. (2010), Mouillet et al. (2011)
hsa-miR-377	PUM2, SOD1, SOD2, ETS1, RNF38, PAK1 STARD7		↓**sPE** Neuroblastoma	Haller et al. (2010), Genovesi et al. (2011)
hsa-miR-409	FGB		Neuroblastoma Medulloblastoma	Zhu et al. (2009), Gattolliat et al. (2011)
hsa-miR-410	CDK1		Neuroblastoma	Haller et al. (2010), Gattolliat et al. (2011), Genovesi et al. (2011)
hsa-miR-411	PPP1CC, USP42, PCDH10, RANBP9	P, F, MP	↓**sPE**	Gattolliat et al. (2011)
hsa-miR-433		P, F, T, MP		Zhu et al. (2009), Miura et al. (2010), Enquobahrie et al. (2011)
hsa-miR-485	NF-KB		Neuroblastoma	Miura et al. (2010)
hsa-miR-487		P, F, MP	Neuroblastoma	Gattolliat et al. (2011)
hsa-miR-494			Preterm labor Medulloblastoma	Miura et al. (2010), Gattolliat et al. (2011), Genovesi et al. (2011)
hsa-miR-495	REDD1, HNF-6, E-cadherin, OC-2		Neuroblastoma Medulloblastoma	Genovesi et al. (2011), Mouillet et al. (2011)
hsa-miR-654	CDKN1A		Neuroblastoma	Gattolliat et al. (2011), Genovesi et al. (2011)
hsa-miR-1247		P	↓**PE**	Gattolliat et al. (2011)

P, predominant expression in placenta tissues; F, first trimester; T, third trimester; sPE, severe pre-eclampsia; PE, pre-eclampsia; MP, maternal plasma.

no differences in the plasma levels of individual miRNAs in women with preeclampsia (Hromadnikova et al., 2012), while other authors reported deregulation up to 22 miRNAs (Yang et al., 2011; Wu et al., 2012). Application of next generation solid sequencing uncovered 15 miRNAs up-regulated and 7 down-regulated in plasma of women with preeclampsia. Among them, eight miRNAs belonging to C19MC were found up-regulated (miR-521, -520h, -517c, -519d, -517d, -542-3p, -518e and -519a) (Yang et al., 2011). A different study showed 13 miRNAs up-regulated and 2 down-regulated in plasma of patients with severe preeclampsia, out of which miR-342-3p (C14MC member) was found up-regulated (Wu et al., 2012). Again, no similarities were observed between the data in both studies.

Despite the fact that no individual miRNA has been confirmed yet as a biomarker for preeclampsia, significant elevation of extracellular miRNAs was found between the 12th and the 16th week of pregnancy in maternal circulation of women who later develop preeclampsia (Hromadnikova et al., 2012). Likewise, analysis of plasma from women with pregnancies complicated with term FGR revealed no differences in the expression of individual miRNAs, but when considered as a group, there was a significant increase in total miRNA levels (Mouillet et al., 2010).

Other pregnancy pathologies are associated with changes in the expression of C19MC and C14MC members. Decreased miR-518b, miR-519c and miR-520h

Table 3
mRNA targets of the human miR-371-3 cluster.

miR	Target	Function of target	Tissue/cell line	References
hsa-miR-372	LATS2	Tumor suppressor	AGS, MCF-7, Tera-1	Enquobahrie et al. (2011)
	TXNIP	Redox regulator, tumor suppressor	Caco-2, HCT-116, SW-620	Voorhoeve et al. (2006), Cho et al. (2009)
	TGFBR2	Tumor suppressor, implicated in Wnt signaling	MCF-7, hESC-like cells	Ragusa et al. (2012)
	P21	G1/S transition	HeLa, Dicer knockdown hESCs	Xu et al. (2009), Subramanyam et al. (2011), Zhou et al. (2011)
hsa-miR-372 hsa-miR-373	NFIB	Oncogene	HepG2 cells	Qi et al. (2009)
	ZEB1	Binding at E-boxes	PC3	Guo et al. (2011)
	BTG1	Anti-proliferative	MCF-7	Mazda et al. (2011)
	LEFTY1	TGFbeta signaling	MCF-7	Zhou et al. (2011)
	DKK1	Blocks Wnt signaling, tumor suppressor	MCF-7, HCT 115 (CRC-cells)	Zhou et al. (2011)
	RECK	Tumor suppressor, essential for mammalian development		Zhou et al. (2011)
	RAD23B ↓	Nucleotide excision repair mechanism	HeLa, MCF-7, HIF-1α, MEF	Loayza-Puch et al. (2010)
hsa-miR-373	LATS2	Tumor suppressor	Huh6, MCF-7, Tera-1	Crosby et al. (2009)
	CD44	Cell–cell interactions, cell adhesion and migration	Clinical breast cancer samples, MCF-7	Voorhoeve et al. (2006), Cairo et al. (2010)
	E-Cadherin –promotor sites	Binding beta-catenin intracellularly → Wnt-signaling	PSC-3, HCT-116 (CRC-cells)	Huang et al. (2008)
	CSDC2 – promotor sites	Cold shock domain-containing protein C2	PSC-3, HCT-116 (CRC-cells)	Place et al. (2008)
has-miR-371-3	Myc	Transcription factor		Place et al. (2008)

concentrations were detected in serum of women with preterm labor when compared with normal pregnancies at term (Montenegro et al., 2009). Expression of miR-517a, miR-517b, miR-518b and miR-519a is significantly reduced in complete hydatidiform mole tissues (Na et al., 2012). An increase in miR-323-3p has been proposed as a novel marker for the early detection of ectopic pregnancy (Zhao et al., 2012). Thus far, no member of the miR-371-3 cluster has been found to discriminate pregnancy disorders (Chim et al., 2008; Gilad et al., 2008; Luo et al., 2009; Miura et al., 2010; Kotlabova et al., 2011). This increasing spectrum of studies and observations highlights the potential of microRNAs as a clinical tool for the diagnosis, treatment and prognosis of a variety of pregnancy disorders.

6. MicroRNA clusters, clinical applications and cancer

Human miRNA genes may function as oncogenes and/or tumor suppressors because they are frequently located at fragile genomic regions involved in cancer (Calin et al., 2004). Pregnancy-related miRNAs are not only expressed in pregnancy associated diseases, but their deregulation can also correlate with the development of several tumors. A recent study in neuroblastoma revealed that most of the recurrent large-scale chromosomal imbalances, including loss of C14q, had impact on the miRNA expression (Bray et al., 2009). Increasing number of studies demonstrate that the chromosome region of C14MC is frequently deleted or genetically altered in hematopoietic and solid tumors, suggesting C14MC as the largest tumor suppressor cluster (Haller et al., 2010; Lavon et al., 2010).

The expression profiles of 180 miRNAs in glial tumors, embryonic stem cells, neural precursor cells and normal adult brain tissues revealed that gliomas have a neural precursor cell-like miRNA signature. C14MC was down-regulated in all tested gliomas, and in addition, low expression of C14MC indicates higher risk of tumor progression (Haller et al., 2010; Lavon et al., 2010). Similarly, the study of 734 human miRNAs and their potential correlation with gastrointestinal stromal tumors identified 44 miRNAs, located in the chromosomal region 14q32.31, which were down-regulated in such tumors (Haller et al., 2010). The relationship between the C14MC expression and the risk of tumor progression has suggested the potential of these miRNAs for clinical application, such as miR-487b and miR-410 which may serve as markers for neuroblastoma diagnosis and prognosis (Gattolliat et al., 2011). Further associations of C14MC miRNAs and human diseases are reviewed in Table 2.

In contrast to C14MC, upregulation of C19MC miRNAs is related with tumor development (Flor and Bullerdiek, 2012). Overexpression of C19MC members has been reported in several cancers: Increase of miR-519d levels in hepatocellular carcinoma cells which correlates with hypomethylation of C19MC can activate cell proliferation and invasion, and impairs apoptosis after doxorubicin treatment (Fornari et al., 2012). Several survival genes are directly targeted by miR-519 in hepatocellular carcinoma cells including CDKN1A/p21, PTEN, AKT3 and TIMP2 (Fornari et al., 2012). Overexpression of miR-519 induced cellular senescence through HuR inhibition (Marasa et al., 2010). Additionally, the C19MC members:

miR-515-5p, miR-518a-3p, miR-520f and miR-525-3p are overexpressed in hepatocellular carcinoma cells. Their serum levels might become useful for improvement of diagnosis in early hepatocellular carcinoma and liver dysplasia (Augello et al., 2012).

Deregulation of C19MC is also present in several cancers within the reproductive system. In breast cancer the C19MC member miR-520c-3p promotes tumor invasion and metastasis (Huang et al., 2008). Similarly, three miRNAs (miR-516a-5p, miR-518c* and miR-519a) are highly expressed in endometrial adenocarcinoma whereas miR-518b is down-regulated in cervical carcinoma (Gilabert-Estelles et al., 2012).

MicroRNA profiling has revealed that miRNAs of the miR-371-3 cluster are, at least partially, up-regulated in several cancers, such as in testicular germ cell tumors (Voorhoeve et al., 2006; Palmer et al., 2010), gliomas (Lavon et al., 2010), hepatoblastoma (Cairo et al., 2010), breast cancer (Huang et al., 2008), thyroid adenomas (Rippe et al., 2010) and colorectal tumors (Bandres et al., 2006). Several tumor suppressors and cell cycle regulators are targeted by members of the miR-371-3 cluster, such as LATS2 (Voorhoeve et al., 2006; Cho et al., 2009; Cairo et al., 2010), TGFBRII (Subramanyam et al., 2011; Zhou et al., 2011), p21 (Qi et al., 2009), BTG1 and LEFTY1 (Zhou et al., 2011), as well as DKK1 as part of a suggested feedback-loop involving Wnt/β-catenin (Zhou et al., 2011). In hypoxic cells, miR-373 increases genomic instability by targeting RAD23B (Crosby et al., 2009) and miR-372 and miR-373 are activated by TWIST and suppress RECK, another potent tumor suppressor (Loayza-Puch et al., 2010). MiR-373 stimulates tumor invasion and metastatic potential in vitro and in vivo by decreasing the expression of CD44 (Huang et al., 2008). E-cadherin expression can not only be influenced by miR-372 and miR-373 via targeting ZEB1 (Mazda et al., 2011), but also by miR-373 binding to complementary sequences in its promoter region (Place et al., 2008). Apart from cancer pathogenesis, miR-371-3 stimulates hepatitis B virus production in a hepatocellular tumor cell line, most likely via targeting nuclear factor I/B (Guo et al., 2011). Targets of the miR-371-3 cluster, their function and association with human diseases are reviewed in Table 3.

7. Conclusion and perspectives

Human pregnancy is accompanied by the expression of a large number of pregnancy-related miRNAs. Many of these miRNAs are physiologically expressed almost exclusively in the placenta or in a few fetal tissues. In humans, they are mostly clustered on chromosome 14 (C14MC) and chromosome 19 (C19MC and miR-371-3 cluster). Numerous members of these clusters can be detected in maternal serum and seem to reflect the state of the placenta and might indicate pregnancy disorders. Therefore, there is a strong potential for these miRNAs to become novel biomarkers in pregnancy. Targeting or mimicking these miRNAs may lead to novel therapeutic concepts. Several pregnancy-related miRNAs have been also detected in correlation with malignant tumors of different origin, and in these situations, may have a similar potential for becoming future biomarkers.

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Elsevier Trophoblast Research Award Lecture: Origin, evolution and future of placenta miRNAs

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ABSTRACT

MicroRNAs (miRNAs) regulate the expression of a large number of genes in plants and animals. Placental miRNAs appeared late in evolution and can be found only in mammals. Nevertheless, these miRNAs are constantly under evolutionary pressure. As a consequence, miRNA sequences and their mRNA targets may differ between species, and some miRNAs can only be found in humans. Their expression can be tissue- or cell-specific and can vary time-dependently. Human placenta tissue exhibits a specific miRNA expression pattern that dynamically changes during pregnancy and is reflected in the maternal plasma. Some placental miRNAs are involved in or associated with major pregnancy disorders, such as pre-eclampsia, intrauterine growth restriction or preterm delivery and, therefore, have a strong potential for usage as sensitive and specific biomarkers. In this review we summarize current knowledge on the origin of placental miRNAs, their expression in humans with special regard to trophoblast cells, interspecies differences, and their future as biomarkers. It can be concluded that animal models for human reproduction have a different panel of miRNAs and targets, and can only partly reflect or predict the situation in humans.

1. Introduction

Placentas from eutherian mammals have developed a wide phylogenetic diversity. They can be roughly categorized in epitheliochorial, endotheliochorial and haemochorial placentas [1]. Even within these groups, however, some major differences remain. For instance, human and mouse placentas belong to the haemochorial type but the human placenta features a range of properties not present in mice with regard to cell biology [2], endocrine system [3], immune system [4] and placental transport [5].

Understanding such interspecies differences is of crucial importance for the elucidation of evolutionary processes. Additionally, there is an enormous practical relevance because animals serve as models for the investigation of pregnancy and its disorders and for toxicological tests, often with limited relevance for humans [6]. Interspecies differences are mostly caused by gene evolutionary mechanisms which include duplication, divergence and reassortment of gene segments [7]. Additionally, gene regulatory mechanisms play a significant role in species divergence. These mechanisms comprise, among others, differences in promoter sequences [8], species-specific transcriptional regulators [9], epigenetic mechanisms such as histone modification [10], DNA methylation [11], and microRNA (miRNA) expression [12].

In recent years, the impact of these regulators has attracted attention. MiRNAs are small, single-stranded molecules of approximately 20–24 nucleotides that are capable of binding to messenger RNAs leading to downregulation of gene expression [13]. Remarkably, human placenta exhibits a specific miRNA expression pattern that dynamically changes during pregnancy [14,15] and is reflected in the maternal plasma [16]. Some of these miRNAs are dysregulated in pregnancy disorders like preeclampsia and intrauterine growth restriction (IUGR), and are potential biomarkers for these pathologies [17–19].

In this review we summarize current knowledge on the origin and expression of human placenta miRNAs, their interspecies differences, and their future as potential biomarkers.

2. Origin and evolution of placenta miRNAs

Creation of biological novelty by genome evolution is an important process that gives rise to new organisms and confers specific characteristics to each phylum. MicroRNAs arose early in evolution and can be found in distantly related organisms like plants and animals. However, the processes of biogenesis and target recognition differ significantly between these kingdoms [13]. When

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analyzing the phylogenetic distribution of animal miRNAs from early on in evolution, only *mir-100* is found to be shared within eumetazoans (all major animal groups except sponges) [20]. Later in evolution, three bursts of miRNA innovation occurred. The first was at the base of the bilaterian lineage, which resulted in 34 miRNAs conserved between deuterostomes (e.g. echinoderms and chordates) and protostomes (e.g. arthropods and mollusks). The second miRNA expansion happened at the base of the vertebrate lineage and the third one occurred in the lineage leading to eutherian (placental) animals which originated most placental miRNAs [12]. As a consequence of these events, there is a correlation between the morphological complexity of a given species and the number of encoded miRNA genes in its genome: according to miRBase version 17, zebrafish genome comprises 358 miRNA genes, mouse 720 and human 1424 [12].

Recognition of homologous sequences within species is a major tool for inferring miRNA functionality, and helps to elucidate the mechanisms involved in their evolution. Since target recognition depends on a perfect Watson-Crick pairing between the mRNA 3′UTR region and the miRNA seed sequence, single nucleotide changes in either of these sequences may alter the function of a miRNA and, consequently, miRNA evolution is strictly related to the evolution of mRNA targets. Frequently, changes in the protein-coding sequences of genes result in a lethal disruption of protein function. Thus, changes in the miRNA target sequences are more desired because they have weaker effects and can contribute to the fine tuning of gene expression without risking protein functionality [21]. The evolutionary pressure that influences natural selection, enforcing conservation or favoring divergence in the sequences of miRNAs:mRNA targets, can be classified into three groups: positive selection (programmed selection), neutral selection and negative selection (anti-targets), depending on how miRNA:mRNA interactions affect the organism [21]. Interestingly, the majority of miRNA targets are not conserved, suggesting neutral or negative selection [21]. Further, it has been shown that mutations in miRNA target sequences occur mostly in the human lineage [22]. As a consequence of these mechanisms, several miRNAs are differentially expressed between species; they can be tissue-restricted and can also vary temporarily within the same tissue [18].

3. Placenta miRNAs, expression and interspecies differences

With the appearance of the placenta in evolution, new miRNAs have emerged, which are not expressed, or only marginally so, in other tissues and organs. To date, more than 500 miRNAs have been reported to be expressed by the human placenta, either in studies from placental tissue [23,24] or isolated trophoblast cells [14,15]. We have previously reported the expression of 762 miRNAs in trophoblast cells isolated from first and third trimester placenta, but only 382 miRNAs were notably expressed ($Ct < 35$) [15]. Several similarities can be observed when comparing miRNA profiles of isolated trophoblast cells and whole placenta tissue. For instance, miR-21, miR-199a, miR-30b, miR-191, miR-24 and miR-30c are among the highest expressed miRNAs in both samples [15,24], suggesting trophoblast cells as the main source of placenta miRNAs.

Further, when comparing first and third trimester trophoblast cells, 31 miRNAs were up-regulated and 14 miRNAs were down-regulated by more than 100-fold [15]. Remarkably, some of these miRNAs belong to three highly expressed clusters: C14MC, C19MC and the miR-371-3 cluster [15]. Changes in the expression of these miRNAs with the gestational age suggest an involvement in the regulation of physiological processes. Here, we review aspects of evolutionary conservation of these clusters among species and of further human-specific miRNAs expressed in the placenta.

3.1. The chromosome 14 microRNA cluster (C14MC)

Comprising 54 identified members, C14MC at the DLK1-DIO3 genomic region on chromosome 14 represents the largest known microRNA cluster in humans [25]. Its origin dates back to a precursor sequence that was amplified by tandem duplication and which remains structurally unchanged among species [18]. C14MC is widely conserved in placental mammals but some species divergences have been reported. In mice, the homologous microRNA cluster is found on chromosome 12 at the DLK1-GTL2 region [26]. However, out of 52 human C14MC miRNAs analyzed on the precursor level, nine lack an ortholog in mice (hsa-miR-432, -654, -300, -1185-1, -1185-2, -889, -655, -541 and -656) [27] (Fig. 1). Similarly, analysis of the mir-379/mir-656 region of C14MC at the genomic level revealed differences between mouse and human in regard to gene conservation. For instance, no evidence for an ortholog in mouse was found for genes of human miR-329-2, -889, -655, -487a and -656. On the other hand, genes for the murine miR-679, -666 and -667 were absent in humans [28]. Similar differences were also observed even between more closely related species, e.g. the gene for miR-654 can be found in mice but not in rats [28].

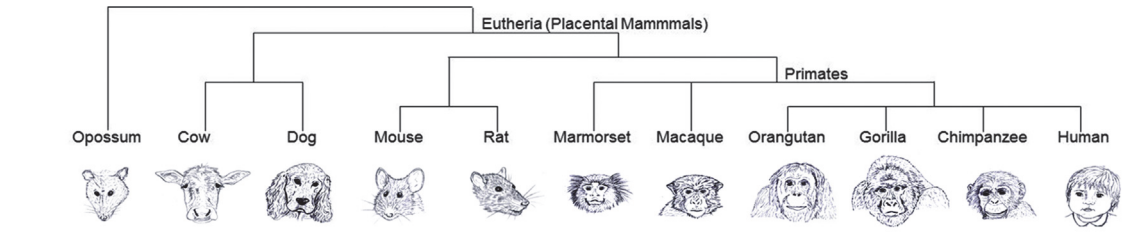
Members of the C14MC are among the highest expressed miRNAs in primary first trimester trophoblast cells and also in the immortalized cell line HTR-8/SVneo [15]. Their expression decreases towards the third trimester and they were almost not detectable in JEG-3 cells or its AC-1 mutant-derived hybrids ACH-3P and AC1-M59 cells [15]. Interestingly, miR-656 that was down-regulated more than 100-fold in third compared to first trimester trophoblast cells [15], belongs to the human miRNAs which do not occur in mice [27,28]. Since dysregulation of this miRNA and other C14MC members is reported to contribute to cancer development [29], it will be interesting to analyze their role in human pregnancy and its pathologies.

3.2. The C19MC and the miR-371-3 cluster

Both these clusters are encoded on chromosome 19 and are predominantly expressed in placental tissue and in stem cells [23,30]. C19MC may be one of the most interesting clusters with regard to placental development during the course of human evolution, because it only appears in primates [31], while orthologs of miR-371-3 cluster can be found in other eutherian mammals including mouse [18].

C19MC consists of 46 miRNA genes which are mostly conserved among primate species [18]. Nevertheless, analysis of C19MC miRNA precursors revealed 13 human miRNAs lacking orthologs in marmoset, and three which are missing in macaque [27] (Fig. 1). C19MC members are strongly expressed in the human placenta [23], and are among the highest expressed miRNAs in third trimester trophoblast cells and in the choriocarcinoma cell lines JEG-3, ACH-3P and AC1-M59 [14,15]. In contrast to the expression of C14MC, C19MC members are poorly expressed in first trimester pregnancy, but their levels increase with gestational age [15,24]. This correlation suggests an association between their expression and the rapid growth and development of the placenta. However, it cannot be assumed that only trophoblast cells are responsible for their expression because new reports have demonstrated expression also in other placental cells, e.g. mesenchymal stem cells [32].

The miR-371-3 cluster is a small cluster consisting of miR-371, -372 and -373. Besides the fact that on the precursor level no orthologs can be found in the mouse [27], this cluster is considered homologous to the mouse cluster miR-290-295 based on the conserved seed sequences [33]. We found expression of the miR-371-3 cluster in primary trophoblast cells and its expression increased slightly in the third trimester. A large difference in



	Opossum	Cow	Dog	Mouse	Rat	Marmorset	Macaque	Orangutan	Gorilla	Chimpanzee	Human
C19MC	No miRNA orthologs	No miRNA orthologs	No miRNA orthologs	No miRNA orthologs	No miRNA orthologs	No orthologs for: hsa-mir-512-2 hsa-mir-515-1 hsa-mir-515-2 hsa-mir-517a hsa-mir-517b hsa-mir-518a-2 hsa-mir-518c hsa-mir-519c hsa-mir-519d hsa-mir-520f hsa-mir-521-1 hsa-mir-521-2 hsa-mir-526a-2	No orthologs for: hsa-mir-518a-1 hsa-mir-518a-2 hsa-mir-520h	All miRNAs have orthologs	All miRNAs have orthologs	All miRNAs have orthologs	
miR-371-3	No miRNA orthologs	No miRNA orthologs	No orthologs for: hsa-mir-372 hsa-mir-373	No miRNA orthologs	No miRNA orthologs	All miRNAs have orthologs	All miRNAs have orthologs	All miRNAs have orthologs	All miRNAs have orthologs	All miRNAs have orthologs	
C14MC	No miRNA orthologs	No orthologs for: hsa-mir-300 hsa-mir-337 hsa-mir-655 hsa-mir-668	No orthologs for: hsa-mir-300 hsa-mir-337 hsa-mir-412 hsa-mir-665 hsa-mir-770 hsa-mir-1247	No orthologs for: hsa-mir-300 hsa-mir-432 hsa-mir-541 hsa-mir-654 hsa-mir-655 hsa-mir-656 hsa-mir-889 hsa-mir-1185-1 hsa-mir-1185-2	No orthologs for: hsa-mir-300 hsa-mir-432 hsa-mir-541 hsa-mir-655 hsa-mir-656 hsa-mir-889 hsa-mir-1185-1 hsa-mir-1185-2	No orthologs for: hsa-mir-136	No orthologs for: hsa-mir-655	All miRNAs have orthologs	No orthologs for: hsa-mir-1247	All miRNAs have orthologs	
Human-specific	No miRNA orthologs	No miRNA orthologs	No miRNA orthologs	No miRNA orthologs	No miRNA orthologs	No miRNA orthologs	No miRNA orthologs	No miRNA orthologs	No miRNA orthologs	No miRNA orthologs	hsa-mir-659 hsa-mir-941 hsa-mir-1302 hsa-mir-3156 hsa-mir-3648 hsa-mir-3673 hsa-mir-3690 hsa-mir-4487 hsa-mir-4739 hsa-mir-5095

Fig. 1. Orthologs of the main human placental miRNA clusters in animals. Information on miRNAs belonging to pregnancy-related C14MC, C19MC and miR-371-3 clusters [18] has been extracted from a recent publication detecting orthologs for all 1733 annotated mature human miRNAs (miRBase version 17) across 11 species by BLAST [27]. Only miRNAs with no orthologs in a specific species are listed. Human-specific miRNAs correspond to those for which no orthologs in any species have been reported. Illustration by Ospina-Prieto.

expression was observed between choriocarcinoma cell lines and the HTR-8/SVneo cell line. JEG-3 cells and their hybrids express the miR-371-3 cluster, while immortalized HTR-8/SVneo do not [15]. These dissimilarities go in line with reported differences in gene and protein expression between these cell lines [34,35]. Besides the known differences in their origin (HTR-8SVneo were immortalized while JEG-3 cells were obtained from choriocarcinoma tissue), the main reason for absence of C19MC and miR-371-3 in HTR-8SVneo cells is not yet known. Nonetheless, as C19MC and miR371-3 are mainly expressed in cells with stemness capacities [30], it may be argued that freshly isolated cytotrophoblast cells and choriocarcinoma cell lines contain a fraction of cells with these capacities, while extravillous trophoblast cells which were the source for HTR8/SVneo do not. Likewise, the function of these clusters remains unclear although recent reports suggest a role in cell maintenance and cell differentiation [15].

3.3. *Human-specific microRNAs*

The search for orthologs of human mature miRNAs in 11 species also revealed 10 miRNAs which are human-specific and 12 further miRNAs with human-specific seed sequences [27] (Fig. 1). Most of these miRNAs exhibit low expression but miR-941 showed remarkable abundance in different tissues [27]. In our hands, miR-941 is expressed in primary trophoblast cells of the first trimester, but was not detectable in those of third trimester. MiR-941 is expressed by JEG-3, AC1-M59 and HTR-8/Svneo cells, but not by ACH-3P cells [15]. The expression of this miRNA in first trimester trophoblast and in cancer cells goes in line with reports about reduced expression of miR-941 upon stem cell differentiation and its potential prominent role in carcinogenesis [27]. Several of the confirmed targets of miR-941 are involved in insulin-associated intracellular signaling pathways, which exist also in trophoblast cells, such as in Wnt signaling, TGF-ß signaling, phosphoinositide-3-kinase, PPAR-gamma and others [27], To our best knowledge, thus far, no studies have been done on specific effects of miR-941 in the placenta.

Further human-specific miRNAs that were detected in trophoblast cells and trophoblast cell lines are miR-572 and miR-1302. While their expression was low in primary first and third trimester trophoblast cells, it was high in all tested cell lines. Remarkably, miR-572 is strongly expressed in the choriocarcinoma cell line JEG-3 [15].

To our knowledge not all known human-specific miRNAs have been investigated in placental tissue or in isolated placental cells. Their expression patterns and role in the evolution of the human placenta and its disorders remain to be investigated.

4. Future of placenta miRNAs as clinical biomarkers

Numerous novel miRNAs have appeared simultaneously with the development of the placenta in evolution. Some of them have

Table 1
Selection of validated targets of potential miRNA biomarkers. PE: Preeclampsia, SGA: Small for gestational age, PTD: Pre-term delivery, GDM: Gestational diabetes mellitus, EP: Ectopic pregnancy.

MicroRNA	Target		Cell type	Ref
PE/SGA/PTD				
miR-210	HSD17B1	Hydroxysteroid (17-beta) dehydrogenase 1	Placenta tissue	[43]
	ISCU	Iron–sulfur cluster scaffold homolog	Swan 71 cells	[39]
miR-517a	AREG	Amphiregulin	Bladder cancer	[51]
	BCLAF1	BCL2-associated transcription factor 1	Bladder cancer	
PE/SGA				
miR-21	PTEN	Phosphatase and tensin homolog	Mz-ChA-1 human cholangiocarcinoma cell lines	[44]
	TPM1	Tropomyosin 1 (alpha)	MCF-7 cells	
	BCL2	B-cell CLL/lymphoma 2	MCF-7 cells	
miR-16	BCL2	B-cell CLL/lymphoma 2	Pituitary adenomas and chronic lymphocytic leukemia tissue	[44]
PE/PTD				
miR-181a	BCL-2	B-cell CLL/lymphoma 2	K562/A02 Leukemia cell line	[52]
	Tcl1	T-cell leukemia/lymphoma 1	Chronic lymphocytic leukemia	[44]
miR-136	AEG-1	Astrocyte elevated gene-1	Glioma	[46]
	Bcl-2	B-cell CLL/lymphoma 2	Glioma	
miR-15b	CCNE1	Cyclin E1	Glioma	[47]
PE/GDM				
miR-222	P57	Cyclin-dependent kinase inhibitor	Hepatocellular carcinoma, breast, prostate and pancreatic cancer	[48]
	FOXO3	Forkhead box O3	Breast cancer cell lines	
	TIMP3	TIMP metallopeptidase inhibitor 3	Gastric cancer	
	MMP1	Matrix metaloproteinase 1	Tongue squamous cell carcinoma	
	SOD2	Manganese superoxide dismutase	Tongue squamous cell carcinoma	
	cKIT	Tyrosine-protein kinase Kit	Cord blood cells	[44]
miR-29a	Insig1	Insulin-induced gene 1	HepG2	[36]
	PCK2	Phosphoenolpyruvate Carboxy Kinase 2	HepG2	
PE/EP				
miR-519d	CDKN1A/p21	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	Hepatocellular carcinoma cells	[53]
	PTEN	Phosphatase and tensin homolog		
	AKT3	V-akt murine thymoma viral oncogene homolog 3 (protein kinase B, gamma)		
	TIMP2	TIMP metallopeptidase inhibitor 2		
PTD				
miR-199a*	COX-2	Cyclooxygenase-2	Osteoarthritis chondrocytes	[54]
miR-338	PTPRT	Protein tyrosine phosphatase, receptor type, T	Neuroblastoma	[55]
miR-449	GMNN	Geminin, DNA replication inhibitor	Gastrin KO mice	[56]
	MET	Met proto-oncogene (hepatocyte growth factor receptor)		
	CCNE2	Cyclin E2		
	SIRT1	Sirtuin 1		
miR-483-5p	ERK1	Extracellular signal-regulated kinase 1	Glioma	[57]
miR-493	RhoC	Ras homolog family member C	Bladder cancer	[58]
	FZD4	Frizzled family receptor 4		
GDM				
miR-132	PTEN	Phosphatase and tensin homolog	Primary neurons and PC12 cells	[49]
	FOXO3a	Forkhead box O3		
	P300	E1A binding protein p300		
PE				
miR-20a	VEGFA	Vascular endothelial growth factor A	Bewo cells	[59]
	EFNB2	Ephrin-B2		
	EPHB4	EPH receptor B4		
	MMP2	Matrix metalloproteinase 2		
	HIF1A	Hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)		
	E2F1	E2F transcription factor 1	HeLa	[44]
miR-155	CCND1	Cyclin D1	Htr8/svneo	[60]
miR-517c	Pyk2	Protein tyrosine kinase 2 beta	Hepatocellular carcinoma	[61]
miR-26b	SLC7A11	Solute carrier family seven member eleven	Human breast cancer MCF7 cells and clinical samples	[62]
miR-1	JCN	Junctin	Cardiomyocytes	[63]
miR-223	GZMB	Granzyme B	Natural killer (NK) cells	[64]
	STAT3	Signal transducer and activator of transcription 3	Macrophage	
	E2F1	E2F transcription factor 1	AML blast	
	FOXO1	Forkhead box O1	Cancer cell lines	
miR-26a	Bcl-2	B-cell CLL/lymphoma 2	Hepatocellular carcinoma cells	[65]
	Mcl-1	Myeloid cell leukemia sequence 1 (BCL2-related)		
	CCND1	Cyclin D1		
	MMP2	Matrix metalloproteinase 2		

been conserved through all placental mammals; others, as the above described, are restricted to smaller or larger groups of species. A major source of placental miRNAs is villous trophoblast, which is able to release miRNA containing exosomes into the maternal circulation suggesting their potential for becoming novel biomarkers for prediction and detection of pathologies in pregnancy [14,24].

Shallow extravillous trophoblast invasion and incomplete endovascular transformation are frequently associated with pregnancy disorders including preeclampsia, gestational diabetes, intrauterine growth restriction and low birth weight [19,36,37]. Preeclampsia is one of the major causes of maternal and perinatal morbidity and mortality worldwide with 5%–8% of all pregnancies being affected by this condition [38,39]. Besides the well-known features such as hypertension and proteinuria, preeclampsia is also associated with impaired implantation, endothelial dysfunction, and systematic inflammation in the mother [38]. Preeclampsia is often associated with intrauterine growth restriction, trophoblast hypoxia and defective placental development or function [40]. Aiming to unravel the role of miRNAs in the development of these diseases, several studies have been carried out comparing miRNA expression in placental tissue or maternal serum of preeclamptic women with that of normal pregnancies. As an attempt to consolidate the information regarding miRNAs in these and other pathologies, we have briefly summarized previously reviewed studies [18,19] and included recent data [38,39,41]. These studies have used samples obtained after delivery from patients with preeclampsia and term-matched normal pregnancies. Only investigations on ectopic pregnancy (EP) and gestational diabetes mellitus (GDM) were performed with serum from patients below 20 weeks of gestation. For estimation of functions of these miRNAs, we have compiled targets involved in the cell cycle, cell proliferation or cell differentiation (Table 1).

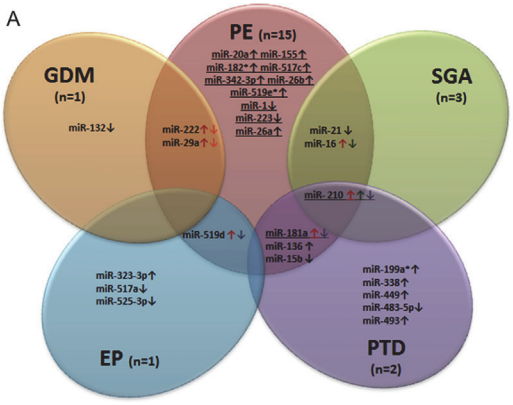
We found 15 reports on miRNAs in preeclampsia but only few studies on small for gestational age (SGA), preterm delivery (PTD), EP and GDM (Fig. 2). Interestingly, data differ between studies and only few miRNAs have been commonly found in at least two investigations (Fig. 2: underlined miRNAs). This may be due to relative small study sizes (5-34 patients), intrinsic patient variations, different definitions of preeclampsia, inclusion criteria and variability in the methodologies, e.g. in the RNA extraction protocols, gene array platforms or statistical analyses. Despite that, some miRNAs are consistently reported dysregulated in different pregnancy pathologies and their further study may be worth pursuing.

In the case of preeclampsia, thus far, 110 miRNAs have been reported to be dysregulated, out of which only 12 are named in at least two independent studies (Fig. 2). Only miR-210 is dysregulated in three different pathologies, which indicates its importance in normal placentation. It is up-regulated in preeclampsia and small for gestational age (reviewed by Ref. [18]), but down-regulated in preterm delivery (PTD) [37]. The physiological role of miR-210 is not yet defined, but growing evidence indicates that up-regulation of miR-210 is associated with altered placental gene expression in response to hypoxic stimuli [39,42]. MiR-210 is expressed predominantly by syncytiotrophoblast. Further, a main target is HSD17B1, a placental steroidogenetic enzyme whose reduction may predict onset of preeclampsia [43] (Table 1). In the trophoblast cell line Swan 71, miR-210 inhibits invasiveness by targeting iron–sulfur cluster scaffold homolog (ISCU) [39]. Altogether, alteration of miR-210 expression may be linked to pregnancy pathologies with shallow placentation like preeclampsia, intrauterine growth restriction and preterm delivery.

Similar to miR-210, miR-21 and miR-16 are dysregulated in preeclampsia and intrauterine growth restriction (Fig. 2). Both miRNAs are considered oncomiRs because they are located in cancer-associated genomic regions, they are able to regulate invasion, and their targets are mainly tumor suppressors [44] (Fig. 2, and Table 1). MiR-21 is the most highly expressed miRNA in trophoblast cells [15] and in placental tissue [24], and its expression is upregulated in JEG-3 cells stimulated with leukemia inhibitory factor (LIF) [45] indicating its involvement in the control of trophoblast proliferation and invasion.

To our knowledge, only two studies on miRNAs in preterm delivery have been published (reviewed by Ref. [19]). The results show three miRNAs (miR-181a, -136 and -15b) dysregulated in placenta tissue from women with PTD as well as in samples of PE patients [19] (Fig. 2). Their potential targets include BCL-2, a protein that regulates cell death [44,46] and cyclin E1 which is involved in cell

A



B

miRNAs	Chr	Sample		No orthologs Ref (29)								
		Tissue	Ref	Chi	Go	Ora	Mac	Mar	Rat	Mou	Dog	Cow	Op
miR-20a	13	PT	(19)										
miR-155	21	PT	(19)										X
miR-182*	7	PT	(19)						X				X
miR-517c	19	PT, S	(18,19)						X	X	X	X	X
miR-342-3p	14	PT, PL	(18, 19,41)										X
miR-26b	2	PT	(19)										
miR-519e*	19	PT	(18,19)						X	X	X	X	X
miR-1	20	PT	(19)		X				X				
miR-223	X	PT, S	(19,41)										
miR-26a-1	3	PT, PL	(19,41)										X
miR-26a-2	12	PT, PL	(19,41)						X				
miR-21	17	PT	(19,41)										
miR-16	3 \ 13	PT	(19)							X			
miR-210	11	PT, PL	(18, 19,39)					X				X	X
miR-181a-1	1	PT, PL	(19)										
miR-181a-2	9	PT, PL	(19)						X				X
miR-136	14	S, CM	(18,19)					X					X
miR-15b	3	PT	(19)										X
miR-199a*-1	19	CM	(19)						X				
miR-199a*-2	1	CM	(19)										
miR-338	17	CM	(19)										
miR-449a	5	CM	(19)										X
miR-449b	5	CM	(19)										
miR-449c	5	CM	(19)				X		X	X	X		X
miR-483-5p	11	PT	(19)	X		X	X		X	X	X	X	X
miR-493	14	PT	(19)	X									X
miR-519d	19	S	(18,19)					X	X	X	X	X	X
miR-323-3p	14	S	(18,19)										X
miR-517a	19	S	(18,19)					X	X	X	X	X	X
miR-525-3p	19	S	(18,19)						X	X	X	X	X
miR-222	X	PT, S	(19)										
miR-29a	7	S	(19)										
miR-132	17	S	(19)	X	X								

Fig. 2. Potential miRNA biomarkers in clinical pathologies and their orthologs in animal models. A) Venn diagram. PE: Preeclampsia, SGA: Small for gestational age, PTD: Pre-term delivery, GDM: Gestational diabetes mellitus, EP: Ectopic pregnancy. MiRNAs underlined were found to be dysregulated in at least two independent studies. Arrows describe up- or down-regulation when compared to normal pregnancies. Overlapping fields display miRNAs affected by all respective pathologies. If two contrary effects were reported, arrows' colors are congruent with that of the respective pathology. "*n*" expresses number of publications reviewed in this manuscript. B) Chromosomal localization, sample origin and lack of orthologs in animal models. PT: Placental tissue, S: Serum, PL: Plasma, CM: Chorioamniotic membranes. Chi: Chimpanzee, Go: Gorilla, Ora: Orangutan, Mac: Macaque, Mar: Marmoset, Rat: Rat, Mou: Mouse, Dog: Dog, Cow: Cow, Op: Opossum.

cycle regulation [47] (Table 1), but the miRNA-target interaction has not been confirmed in placenta.

In the development of gestational diabetes mellitus (GDM), the role of miRNAs remains largely unexplored. A recent publication shows that miR-29a, miR-132 and miR-222 were significantly down-regulated in GDM patients [36]. MiR-29a targets Insig1 and PCK2, which are relevant molecules for gluconeogenesis and fatty acid metabolism. Thus, miR-29 may be a negative regulator of serum glucose and a novel candidate biomarker for predicting GMD [36]. The role of miR-222 remains unclear because it depends on the cellular context: miR-222 can act as an oncomir regulating p57, FOXO3 and TIMPs, but also can inhibit invasion by reducing MMP-1 and SOD2 [48]. Finally, miR-132 in primary neurons and PC12 cells controls cell survival by direct regulation of PTEN, FOXO3a and P300 [49].

Study of ectopic pregnancy revealed four miRNAs dysregulated when compared with spontaneous abortion and viable intrauterine pregnancy [50] (Fig. 2). Among them, miR-323-3p was significantly increased in ectopic pregnancy, and its expression predicted these cases with a sensitivity of 37% and a specificity of 90%. Combined measurement of hCG, progesterone and miR-323-3p levels was able to discriminate between ectopic and normal pregnancy with a sensitivity of 93% and a specificity of 72% indicating a great diagnostic potential [50].

Since animal models frequently are employed for the study of pregnancy pathologies, we decided to investigate the homologous precursor sequences of the above described miRNAs. Besides members of the placenta-specific miRNA clusters, some other miRNAs with potential as biomarkers lack orthologs in models widely used like mouse (miR-16, -449c, and -483-5p), rat (miR-182*, -1, -26a-2, -181a-2, -199a*-1, -449c and -483-5p), and even chimpanzee (miR-483-5p and -132) (Figs. 1 and 2). Lack of homologous sequences in animals may suggest that those miRNAs play a role in the development of human-specific pregnancy pathologies.

5. Conclusions

The above reviewed studies indicate the future usage of miRNAs as biomarkers for pregnancy disorders. Several of these miRNAs do not have orthologs in species widely used as experimental models (Figs. 1 and 2). Therefore, it can be concluded that the use of animal models in miRNA research may provide results often not relevant for humans or not predicting their functions in human pregnancy. Due to the different miRNA expressed in non-human species, but also due to the enormous differences in target sequences, it is hard to estimate the extent of transferability of animal models to humans. Human *ex vivo* and *in vitro* models may more closely display human physiology and pathology than animals can do.

Conflict of interest statement

The authors declare that there is no conflict of interest regarding the publication of this article.

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miR-21 regulates trophoblast cell functions by targeting phosphatase and tensin homologue (PTEN) and programmed cell death 4 (PDCD4)

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Abstract

Background: Deregulation of miRNA expression in the placenta has been associated with pregnancy complications such as preeclampsia. miR-21 is highly expressed in trophoblast cells, and its dysregulation is linked to various cancers and pregnancy pathologies. We aim to characterize miR-21 functions and targets in trophoblastic cell lines.

Methods: miR-21 was inhibited or overexpressed by transfection with miR-21-antagomir or – mimic in the trophoblastic cell lines JEG-3 and HTR-8/SVneo. Cell proliferation was measured by a colorimetric BrdU assay and invasion by a Matrigel assay. Migration was assessed by Transwell and scratch wound-healing assays. Apoptosis was determined by flow cytometry and Western blotting. Expression of the potential miR-21 targets phosphatase and tensin homolog (PTEN) and programmed cell death 4 (PDCD4), as well as phosphorylation of AKT was analyzed by Western blotting.

Results: Constitutive expression of miR-21 was higher in HTR-8/SVneo cells than in JEG-3 cells. Inhibition of miR-21 decreased cell proliferation, migration, and invasion in JEG-3 and HTR-8/SVneo cells and additionally induced apoptosis in JEG-3 cells. Silencing of miR-21 enhanced PDCD4 expression in JEG-3 cells, and PTEN expression in HTR-8/SVneo cells. Inhibition of miR-21 significantly increased phosphorylation of AKT in HTR-8/SVneo cells.

Conclusion: miR-21 is expressed by both cell lines but at different levels. MiR-21 is involved in regulating cell growth, migration, invasion and apoptosis in trophoblastic cells, potentially by controlling the intracellular signaling via PTEN in HTR-8/SVneo

cells and PDCD4 in JEG-3 cells. Dysregulation of miR-21 might be associated with pregnancy disorders suggesting its potential as novel biomarker.

Key words: trophoblast, microRNA, miR-21, invasion, proliferation, apoptosis

Introduction

Multiple processes are involved in normal implantation and placental development for the establishment of pregnancy [1]. Invasion of extravillous trophoblast cells (EVTs) is a key step of implantation and is tightly regulated by several cytokines, growth factors, enzymes and miRNAs [2]. Dysregulation of EVT invasion is a leading cause for pregnancy complications such as preeclampsia and fetal growth restriction [3].

miRNAs are small (18-24 nucleotides) non-coding RNAs that control gene expression by inhibition of translation and induction of transcript degradation [4,5]. They can regulate physiological and pathological processes such as embryo development, cell differentiation, cell proliferation, apoptosis, angiogenesis, inflammation and cancer [6,7]. Therefore, miRNAs have been studied as potential biomarkers for clinical application [8,6].

The human *MIR-21* gene is located at on chromosome 17q23.2 overlapping with the transmembrane protein 49 coding gene *TMEM49* [9]. MiRNA-21 is recognized as an oncomir and has been reported to be up-regulated in various types of cancer such as breast, gastric, lung, prostate and cervical cancer [10,11]. Several target genes of miR-21 have been experimentally validated including programmed cell death 4 (PDCD4), phosphatase and tensin (PTEN) homologue, BCL2, tissue inhibitor of metalloproteinases 3 (TIMP3), Fas ligand (FasL) and others [12]. Among them, PTEN and PDCD4 are known as tumor suppressor genes and play important roles in controlling cell proliferation, apoptosis, invasion and metastasis [13,12].

Although it is well known and documented that miR-21 is up-regulated in cancer, only little is known about miR-21 functions in reproduction and development, especially in the placenta. Recent studies have demonstrated that the human

placenta expresses high levels of miR-21 [14], and low miR-21 levels are associated with low birth-weight [15]. However, the specific role and the molecular pathways regulated by miR-21 in implantation and placentation are not yet known.

Previously, we have exposed that miR-21 is one of the highest expressed miRNAs (out of 762 miRNAs) in isolated first trimester trophoblast cells and also in the immortalized first trimester trophoblast cell line HTR-8/SVneo [14]. In the present study, we demonstrate the effects of miR-21 on trophoblastic cell functions as well as the elucidation of two potential targets PTEN and PDCD4 by experimental inhibition and overexpression. Our experimental design includes the use of two trophoblastic cell lines: the choriocarcinoma cell line JEG-3 and the immortalized human first trimester extravillous trophoblast cell line HTR-8/SVneo [16]. The use of cell lines as models instead of primary trophoblast cells is due to the lack of proliferation, short lifespan and limited siRNA transfectability of the latest ones [17,18].

Materials and methods

Cell lines and cell culture

The immortalized human trophoblast cell line HTR-8/SVneo (kind gift from CH Graham, Kingston, Canada) was cultured in RMPI-1640 medium (PAA Laboratories, Pasching, Austria). The choriocarcinoma cell line JEG-3 (DSMZ, Braunschweig, Germany) was cultured in Ham F-12 medium (PAA Laboratories). Both media were supplemented with 10% heat-inactivated fetal bovine serum (Sigma, Steinheim, Germany), 50 U/ml penicillin and 50 µg/ml of streptomycin (PAA Laboratories). Cells were maintained under standardized conditions (37°C, 5% CO2, humidified atmosphere) and regularly screened for absence of mycoplasma.

Transfection with miR-21 mimic and antagomir

Cells were seeded in 6-well plates in antibiotic-free medium overnight to reach 30-50% confluence at the time of transfection. Transfection was performed using Oligofectamine (Invitrogen life technologies, Darmstadt, Germany) according to the manufacturer's instructions. MiR-21 antagomir (anti-miR-21) for the silencing, miR-21 mimic (pre-miR-21) for overexpression, and the respective negative controls were obtained from Thermo Fisher Scientific (Schwerte, Germany). During 48 h after transfection, cells were cultured in medium before initiation of analyses. Expression levels of miR-21 were analyzed by real time qPCR. Cells were stimulated with 1 ng/ml epidermal growth factor (EGF) (Millipore, Schwalbach, Germany) for 5 min to analyze AKT phosphorylation.

RNA isolation

Total RNA was extracted using TRIzol reagent (Invitrogen life technologies) according to the manufacturer's instructions. Total RNA concentration was determined at a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). Samples with A260/A280 ratio greater than 1.8 were stored at −80°C until being processed.

Quantification of miRNA by quantitative RT–PCR (qRT–PCR)

Expression levels of miR-21 were determined by reverse transcription using Taqman miRNAs reverse transcription kit (Applied Biosystems, Damstadt, Germany). Quantitative real time PCR was performed using Taqman microRNA assay (hsa-miR-21, Assay ID: 000397; RNU-48, Assay ID: 001006) and Taqman universal PCR master mix reagents (Applied Biosystems). Quantitative PCR was run on a 7300 Real-time PCR System (Applied Biosystems). Expression of miR-21 was normalized using the 2-ΔΔCt method relative to RNU48. Experiments were performed in triplicates and expressed as mean ± standard error (SE).

Western blot analyses

Cells were washed in PBS and lysed using RIPA lysis buffer (1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 150 mM NaCl and 50 mM Tris–HCl) containing protease inhibitors, followed by three freeze-thaw cycles. Total protein concentrations were assessed by the Bradford method (Sigma). 30 µg of protein extract was loaded on a 10% SDS-PAGE, and resolved proteins were transferred to a nitrocellulose membrane (Hybond-P; GE Healthcare, Freiburg, Germany). Non-specific binding sites were blocked by incubation with TBST containing 5% (w/v) non-fat dried milk for

1 h at room temperature. Membranes were immunoblotted with the respective specific antibody (1:1000 dilution) overnight at 4 °C, followed by 1h incubation at room temperature with the HRP-conjugated anti-rabbit IgG secondary antibody (1:10,000 dilution). The primary monoclonal antibodies rabbit anti-human PTEN, rabbit anti-human PDCD4, rabbit anti-human AKT, rabbit anti-human phospho-AKT (p-AKT), rabbit anti-human caspase-3, rabbit anti-human ß-actin and HRP-conjugated anti-rabbit IgG were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Blots were developed using an enhanced chemiluminescence (ECL) detection kit (Millipore, Schwalbach, Germany). Intensity of Western blot bands was analyzed and quantified by a MF-ChemiBis 3.2 gel documentation system and Totallab TL100 software version 2006 (Biostep GmbH, Jahnsdorf, Germany) and normalized to ß-actin.

Proliferation assay

Proliferation was assessed using a colorimetric BrdU-incorporation ELISA (Roche Applied Science, Mannheim, Germany). 48 h after transfection, cells were seeded in 96 well plates at the density of $5x10^3$ cells per well. After further 24, 48 and 72 h cultivation, cells were incubated in fresh BrdU-containing medium for another 2 h. The colorimetric ELISA for BrdU quantification was performed following the manufacturer's instruction.

Matrigel invasion and migration assay

Cell invasion and migration were evaluated in 24-well inserts with 8 µm pore size Transwell membranes (Millipore). Suspensions of $1x10^5$ cells were seeded in the inserts pre-coated with Matrigel (BD Biosciences, Heidelberg, Germany). After 24 h

incubation at 37°C in a 5% CO_2 atmosphere, cells on the upper chamber were removed using cotton swab, while invading cells were fixed with chilled 80% ethanol and stained with 0.1% crystal violet. After washing, cells were de-stained by adding 1% acetic acid. Colorimetric absorbance was detected at 570 nm and normalized to non-treated cells. Cell migration assay was performed by using the same protocol without Matrigel.

Wound healing assay

The method was performed as previously described [19]. After transfection, cells were cultured in 6 well plates until confluence. A pipette tip was used to remove cells from the bottom by scratching to create “wounds”. Cells were then rinsed with serum free medium several times to remove free floating cells and debris. Fresh medium containing low concentration (1%) of FBS was added to prevent proliferation. Serial photographs were taken at 0, 24, and 48 h. A total of six wounded areas were selected randomly from each well and the cells in three wells of each group were quantified. The widths of the “wound” (scratch areas) were measured by ImageJ software (NIH, http://rsbweb.nih.gov/ij/).

Analysis of apoptosis and necrosis

Quantification of apoptotic cells was performed by flow cytometry. Cells were harvested and washed twice with cold PBS. Cells were stained with FITC annexin V and propidium iodide (PI) (Immunotools, Friesoythe, Germany) for 15 min and then measured at a FACS Calibur and analyzed by using Cell Quest software (both BD

Biosciences). In 10,000 cells, percentages of apoptotic and necrotic cells were determined. Annexin V-positive/PI-negative cells were designated as apoptotic and Annexin V-positive/PI-positive cells as necrotic. Each experiment was performed in triplicates (simultaneous cultures in three different wells). Additionally, Western blots have been performed for detection of caspase-3 and its cleaved isoform, an effector of the caspase-dependent apoptosis cascade.

Bioinformatic prediction of miR-21 target genes

Potential miR-21 targets were selected after searching in the bioinformatics platforms TargetScanHuman (V6.2) (http://www.targetscan.org), PicTar (http://pictar.mdc-berlin.de), and miRecords (http://mirecords.biolead.org/).

Statistical analysis

Each experiment was repeated independently at least 3 times, and each has been performed in 3 simultaneous biological replicates. Values were expressed as means ± SE. Statistical analyses were performed by two-tailed Student's *t* test. *p* values < 0.05 were considered statistically significant.

Results

Expression of miR-21 after transfection with mimic and antagomir

As assessed by semi-quantitative RT-PCR, miR-21 was expressed in both analyzed cell lines, 7-fold higher in HTR-8/SVneo cells than in JEG-3 cells (Fig.1A). Overexpression of miR-21 resulted in approximately 770-fold increased expression in JEG-3 cells and 50-fold increase in HTR-8/SVneo cells (Fig.1B). Silencing of miR-21 reduced its expression in JEG-3 cells by 50% and in HTR-8/SVneo cells by 70% (Fig.1C).

miR-21 regulates cell proliferation, invasion and migration

Cell proliferation as determined by BrdU incorporation was significantly inhibited in HTR-8/SVneo and JEG-3 cells after miR-21 silencing (Fig.2A, upper panels) and significantly increased after miR-21 overexpression (Fig.2A lower panels).
MiR-21 overexpression enhanced cell invasion, whereas silencing of miR-21 resulted in decreased invasion of JEG-3 and HTR-8/SVneo cells (Fig. 2B).
Overexpression of miR-21 also significantly increased cell migration in HTR-8/SVneo but not in JEG-3 cells (Fig. 3A), while miR-21 silencing significantly suppressed migration in both HTR-8/SVneo and JEG-3 cells (Fig. 3A).

Additionally, a scratch wound healing assay has been performed to further demonstrate the function of miR-21 in migration potency. MiR-21 silencing significantly inhibited wound closing in HTR-8/SVneo and JEG-3 cells (Fig. 3B and

3C left panels). In contrast, miR-21 overexpression significantly promoted migration of HTR-8/SVneo cells and JEG-3 after 48h (Fig. 3B and 3C right panels). In both cases, the effect was less evident in JEG-3 than in HTR-8/SVneo cells.

Silencing of miR-21 induces cell apoptosis

Forty-eight hours after miR-21 silencing, the percentage of apoptotic and necrotic cells has been assessed by annexin V/PI staining. Apoptosis significantly increased in miR-21 silenced JEG-3 cells, but not in HTR-8/SVneo cells (Fig.4A). The percentage of necrotic cells remained constant in both cell lines after silencing of miR-21 (Fig.4A).

As shown by Western blotting (Fig. 4B), caspase-3 levels increased in JEG-3 cells after miR-21 silencing but the cleaved isoform was not significantly changed. This effect was not observable in HTR-8/SVneo cells (data not shown). Both assays indicate that down-regulation of miR-21 promotes apoptosis in JEG-3 cells, but not in HTR-8/SVneo cells.

Prediction of miR-21 targets

Putative miR-21 targets were identified by the bioinformatics platforms TargetScanHuman (V6.2) (http://www.targetscan.org), PicTar (http://pictar.mdc-berlin.de), and miRecords (http://mirecords.biolead.org/). Among others, PTEN and PDCD4 were recently reported to have miR-21 binding sites within their 3'-UTR and were confirmed as targets of miR-21 in other cell models [20,21]. Since both proteins

play important roles in the control of cell survival and invasion, we selected them as potential miR-21 targets for this study.

miR-21 targets differentially PDCD4 and PTEN in trophoblastic cells

After transfection of JEG-3 cells with anti-miR-21, PDCD4 protein expression increased remarkably, while transfection with pre-miR-21 resulted in a significant decrease in PDCD4 levels as assessed by Western blotting (Fig.5A, left panel). These effects were not observable in HTR-8/SVneo cells (Fig. 5A, right panel).

In contrast, anti-miR-21 transfected HTR-8/SVneo cells showed elevated PTEN expression, whereas transfection with pre-miR-21 reduced PTEN levels (Fig. 5B, left panel). There were no significant differences of PTEN expression after inhibition or overexpression of miR-21 in JEG-3 cells (Fig.5B, right panel).

Upon EGF stimulation, p-AKT was significantly increased in HTR-8/SVneo cells treated with pre-miR-21 and significantly decreased in cells treated with anti-miR-21 when compared to controls (Fig. 5C).

Discussion

miRNAs regulate genes at post-transcriptional level and play an essential role in biological and pathological conditions including cell differentiation, cell growth, migration, invasion, apoptosis and carcinogenesis [6,9]. MiRNAs have also been proven to play an important role in the development of normal pregnancy by regulating trophoblast cell functions [2,22,14].

miR-21 is considered an oncogenic miRNA (oncomiR) due to its wide implication in initiation and progression of human tumors [9]. Experimental data revealed that miR-21 regulates cell functions by anti-apoptotic and pro-survival mechanisms in several cell types [23,24]. Remarkably, miR-21 is not only expressed in cancer cells, but also in maternal plasma, placenta tissue and trophoblast cells [15,25,14]. These observations suggest that miR-21 may act as regulator of trophoblast functions but its specific functions are still largely unknown. In this study, we aimed to identify the function and targets of miR-21 in two trophoblastic cell lines that resemble trophoblast cells of first and third trimester.

The HTR-8/SVneo cell line has been established after simian virus induced immortalization of first trimester extravillous trophoblast cells [26], while JEG-3 cells derive from a third trimester human choriocarcinoma metastasis [27]. Our results demonstrate that miR-21 is higher expressed in HTR-8/SVneo cells than in JEG-3 cells. This result coincides with our previous observation that miR-21 is amongst the highest expressed miRNAs in first trimester trophoblast cells whose expression decreases in third trimester trophoblast cells [14]. During first trimester of pregnancy, trophoblast cells are highly proliferative and invasive, which decreases towards third trimester [28]. This appears to be also resembled by HTR-8/SVneo cells which exhibit higher invasiveness than JEG-3 cells [29]. Thus, we further investigated the effects of overexpression and silencing of miR-21 on cell proliferation, migration and invasion of these trophoblastic models.

Overexpression of miR-21 resulted in increased cell proliferation, migration (in the wound healing assay) and invasion in both cell lines, while abrogation of miR-21 by treatment with antagomir resulted in significant decrease of these properties. These observations are consistent with previous studies showing that miR-21 regulates cell

proliferation, migration and invasion in several cell types including endothelial cell, smooth muscle cell and cancer cells [30,21].

Only in JEG-3 cells, inhibition of miR-21 led to enhanced induction of caspase-3 expression and apoptosis. These effects were not observed in HTR-8 cells suggesting that miR-21 may have partly different functions and targets in different trophoblastic cell lines.

PTEN and PDCD4 have been validated as targets of miR-21 in various cell types Both proteins are implicated in cell proliferation, migration, invasion and apoptosis in several cell types [12,31,32]. Previous studies have reported the essential role of PTEN in embryonic development and trophoblast invasion during placentation [33,31]. We have investigated whether miR-21 controls trophoblast cell functions via regulating PDCD4 and PTEN.

Our results show that PDCD4 is negatively correlated with miR-21 expression, and thus being a potential target of miR-21 only in JEG-3 but not in HTR-8/SVneo cells. Subsequently, also expression of caspase-3 and apoptosis correlate negatively with miR-21 only in JEG-3 cells. It has been reported previously that PDCD4 is essential for caspase-3 activation and that miR-21 induces apoptosis via PDCD4/caspase-3 pathway [34].

In contrast to JEG-3 cell, in HTR-8/SVneo cells miR-21 expression is correlated negatively with PTEN. PTEN functions as an antagonist of the AKT pathway by dephosphorylation of phosphatidylinositol-3,4,5-trisphosphate ($PI(3,4,5)P_3$), leading to inactivation of AKT [35]. As an effector of the PI3K pathway, AKT can be activated by EGF stimulation. In our hands, miR-21 expression levels correlates positively with AKT phosphorylation after EGF treatment, demonstrating its function via the PTEN/AKT pathway, which is implicated in trophoblast cell invasion [33]. Altogether,

the results are consistent with previous studies in a variety of cell systems indicating that miR-21 regulates cell proliferation, migration, invasion and apoptosis by targeting PTEN and PDCD4 [36-39].

Conclusion

miR-21 regulates proliferation, apoptosis, migration and invasion in trophoblastic cells. This occurs in HTR8/SVneo cells by targeting PTEN and under involvement of the AKT pathway and in JEG-3 cells by targeting PDCD4 (Fig.6). It is not clear if this difference is due to the different placental origin of both cell lines (e.g. first trimester versus third trimester), due to the different immortalization mechanisms (virus versus tumor) or due to other reasons. Nevertheless, the similar functions in the different systems, including in the trophoblast derived cell lines, allows the assumption of a similar role in cytotrophoblast cells *in vivo*. The high expression and the fundamental functions of miR-21 in the human placenta insinuate that its dysregulation may be associated with pregnancy pathologies, which requires further investigations.

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Figure Legends

Figure 1. miR-21 expression in HTR-8/SVneo and JEG-3 cells.

A) miR-21 basal expression determined by qPCR and calculated using 2^{-dCt} method normalized to RNU48. B) miR-21 relative expression in cells transfected with pre-miR-21 (20 nM) for overexpression, or C) anti-miR-21 (120 nM) for silencing. MiR-21 expression was calculated using 2^{-ddCt} method normalized to RNU48 and relative to non-transfected cells. Data is represented as mean ± SE, n=3 each in triplicates. *p<0.05.

Figure 2. Effect of miR-21 silencing and overexpression on cell proliferation and invasion. Cells were transfected with either anti-miR (120 nM) for silencing or pre-miR (20 nM) for overexpression for 48 h before being seeded for functional assays. **A)** BrdU time-course assay showing cell proliferation in transfected HTR-8/SVneo and JEG-3 cells. **B)** Cell invasion analysed by Matrigel assay for 24 h. Data is represented as mean ± SE, n=3 each in triplicates. *p<0.05 compared to control.

Figure 3. Effect of miR-21 silencing and overexpression on JEG-3 and HTR-8/SVneo cell migration. Cells were transfected with anti-miR-21 (120 nM), pre-miR-21 (20 nM), or the respective controls for 48 h before being seeded for functional assays. **A)** The number of migrative cells after 24 h was determined using Transwell chamber without matrigel and normalized to non-transfected cells. **B)** Wound-healing assay showing migration of transfected HTR-8/SVneo and **C)** JEG-3 cells. Repopulation of the cell free area was measured up to 48 h. In each well, six cell free

areas were photographed (x40) and repopulation was quantified by ImageJ software. The bars represent mean ± SE of n=3 each in triplicates. *p<0.05).

Figure 4. miR-21 influences apoptosis only in JEG-3 cells. Cells were transfected with either anti-miR-21 (120 nM) or -control for 48 h. **A)** Apoptosis and necrosis were quantified by FACS after Annexin V-FITC and PI labelling. Left panel: Representative FACS data of JEG-3 and HTR-8/SVneo cells after transfection. Right panel: Bar graph represents the mean of apoptosis and necrosis cells relative to non-transfected cells (n=3). **B)** Representative Western blotting analysis of caspase-3, cleaved-caspase-3 and β-actin in JEG-3 cells after 48 h of transfection (left panel). Expression levels were assessed by densitometry and normalized to non-transfected cells (right panel). Bars represent mean ± SE, n=3 each in triplicates. *p<0.05.

Figure 5. miR-21 targets PDCD4 in JEG-3 cells and the axis PTEN/AKT in HTR-8/SVneo cells. HTR8/SVneo and JEG-3 cells were transfected with anti-miR-21, pre-miR-21 or controls for 48 h, and stimulated or not with EGF. Proteins were extracted and subjected to SDS-PAGE and Western blotting for PDCD4, PTEN, p-AKT, AKT and β-actin. Controls treated with only medium and controls treated with non-genomic RNA sequences have been performed in all experiments and gave no significant differences (data not shown). Representative Western blots and densitometric analysis of **A)** PDCD4, **B)** PTEN. **C)** Western blot and analysis of p-AKT and AKT expression after stimulation with 1 ng/ml EGF for 5 min. p-AKT relative expression was assessed as p-AKT/AKT and normalized to pre-miR-control cells .

Figure 6. A schematic proposed of miR-21 function in trophoblastic cell lines. miR-21 controls proliferation, migration, invasion and apoptosis in trophoblastic cells but different intracellular mechanisms. In the immortalized first trimester trophoblast HTR-8/SVneo cell line, miR-21 downregulates PTEN, and subsequently, inhibits the dephosphorylation of PIP3 into PIP2 resulting in AKT activation. In JEG-3 choriocarcinoma cells, miR-21 downregulates PDCD4 and thereby, influences various cellular responses.

Figures

Figure 1

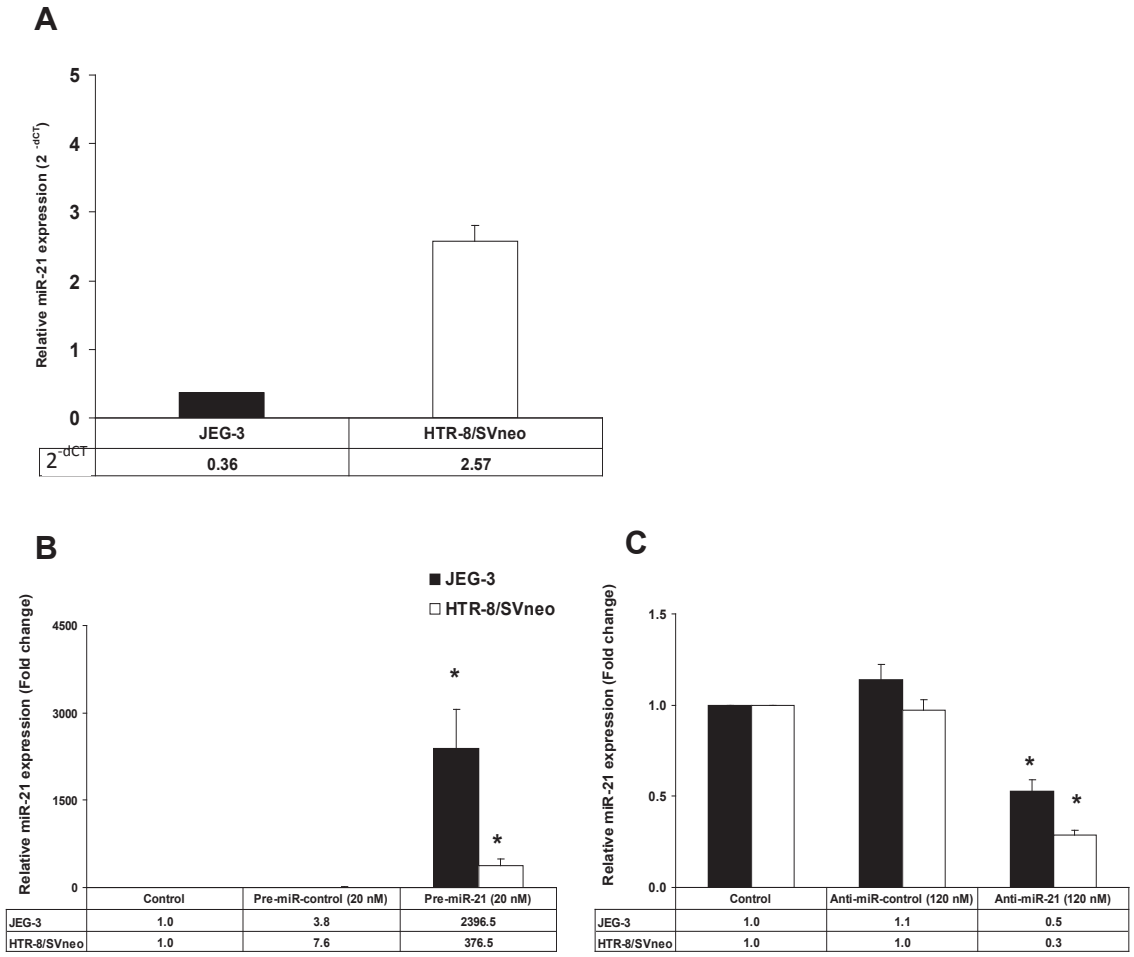
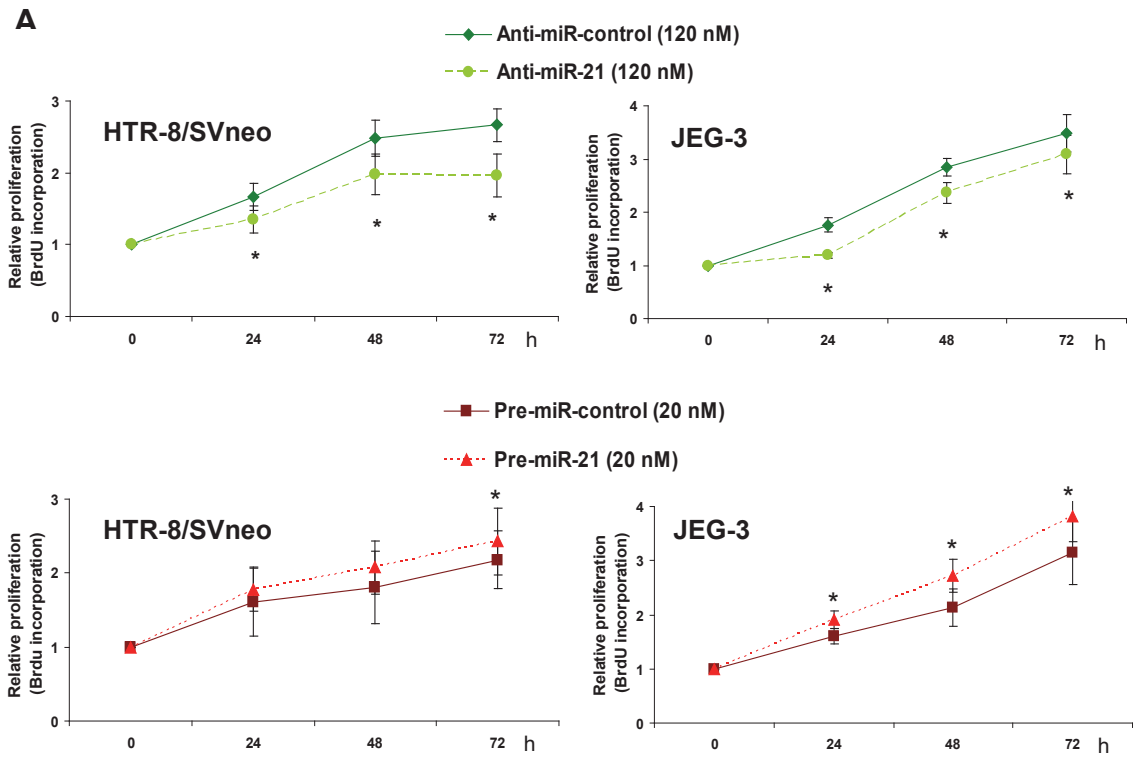


Figure 2


A
Anti-miR-control (120 nM)
Anti-miR-21 (120 nM)
HTR-8/SVneo
JEG-3
Relative proliferation (BrdU incorporation)
h
Pre-miR-control (20 nM)
Pre-miR-21 (20 nM)
HTR-8/SVneo
JEG-3
Relative proliferation (Brdu incorporation)
h

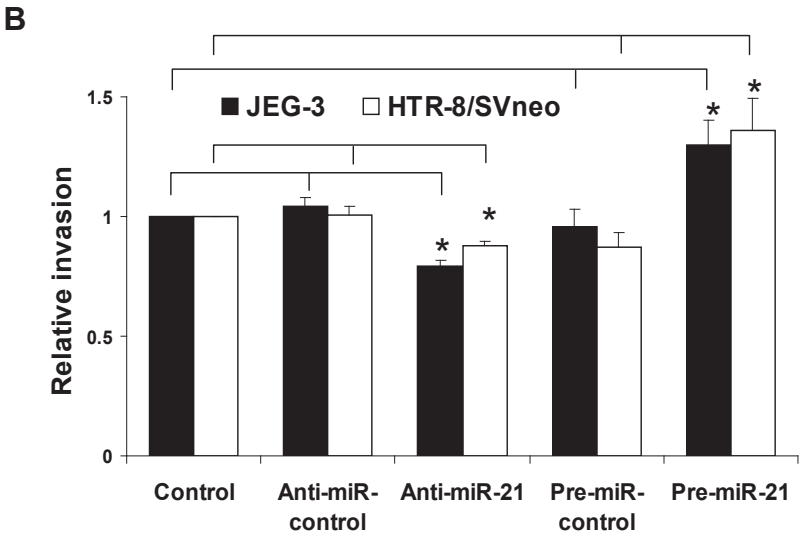
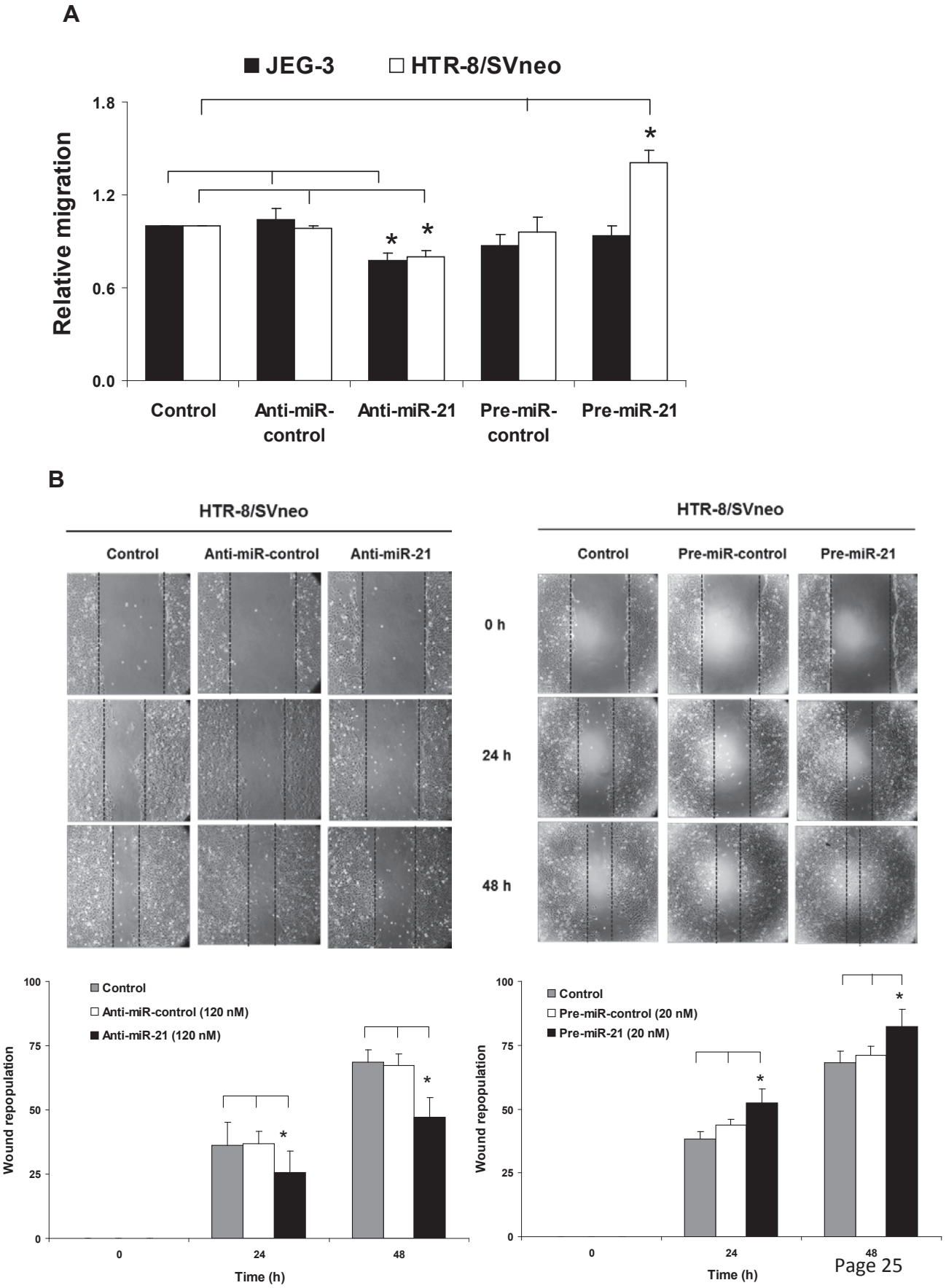

B
JEG-3
HTR-8/SVneo
Relative invasion
Control
Anti-miR-control
Anti-miR-21
Pre-miR-control
Pre-miR-21

Figure 3


A
JEG-3
HTR-8/SVneo
Relative migration
1.8
1.2
0.6
0.0
*
*
*
Control
Anti-miR-control
Anti-miR-21
Pre-miR-control
Pre-miR-21
B
HTR-8/SVneo
Control
Anti-miR-control
Anti-miR-21
HTR-8/SVneo
Control
Pre-miR-control
Pre-miR-21
0 h
24 h
48 h
Wound repopulation
Control
Anti-miR-control (120 nM)
Anti-miR-21 (120 nM)
100
75
50
25
0
0
24
48
Time (h)
Wound repopulation
Control
Pre-miR-control (20 nM)
Pre-miR-21 (20 nM)
Time (h)

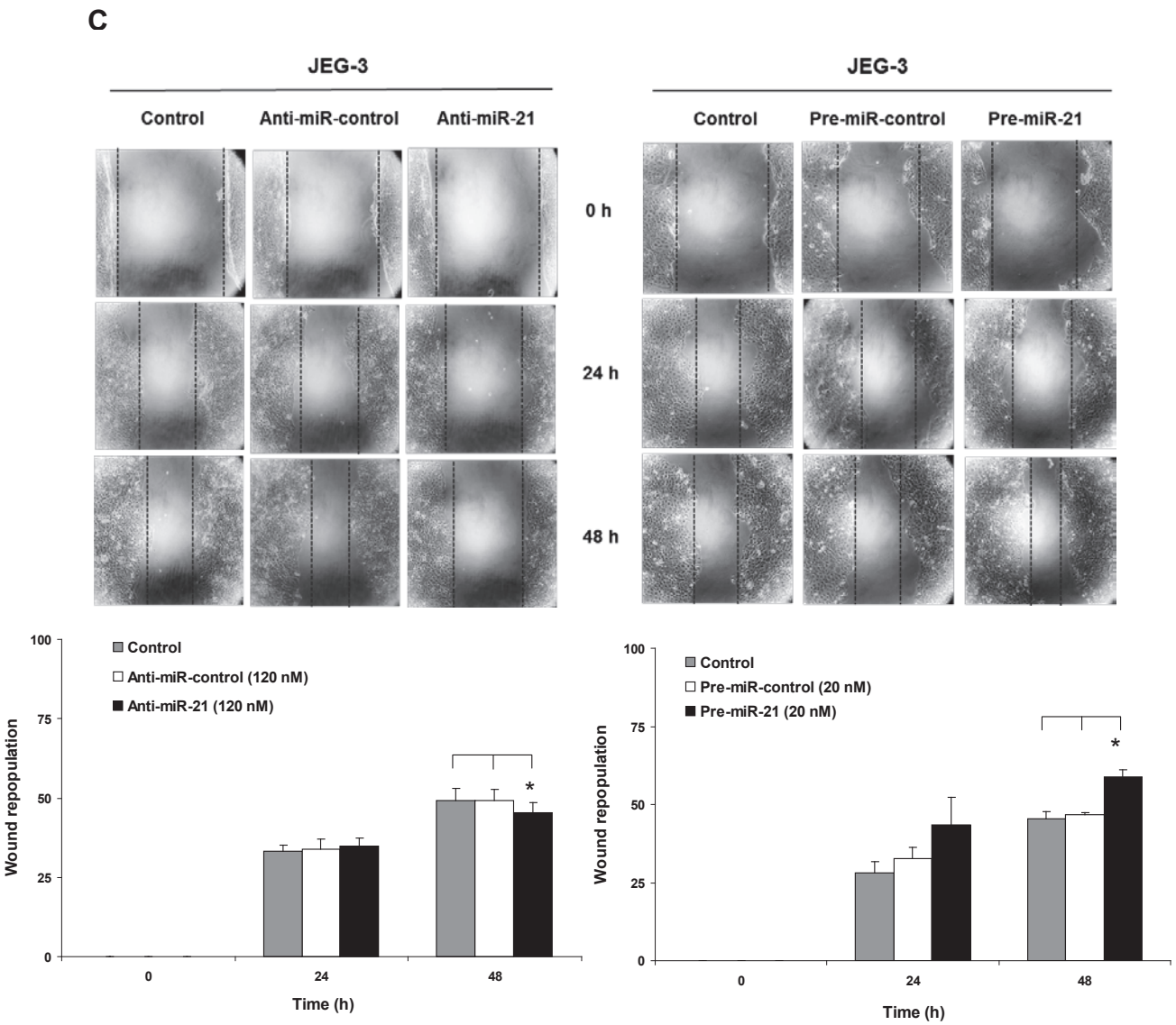

C
JEG-3
Control
Anti-miR-control
Anti-miR-21
JEG-3
Control
Pre-miR-control
Pre-miR-21
0 h
24 h
48 h
Control
Anti-miR-control (120 nM)
Anti-miR-21 (120 nM)
Wound repopulation
100
75
50
25
0
0
24
48
Time (h)
*
Control
Pre-miR-control (20 nM)
Pre-miR-21 (20 nM)
Wound repopulation
100
75
50
25
0
0
24
48
Time (h)
*

Figure 4

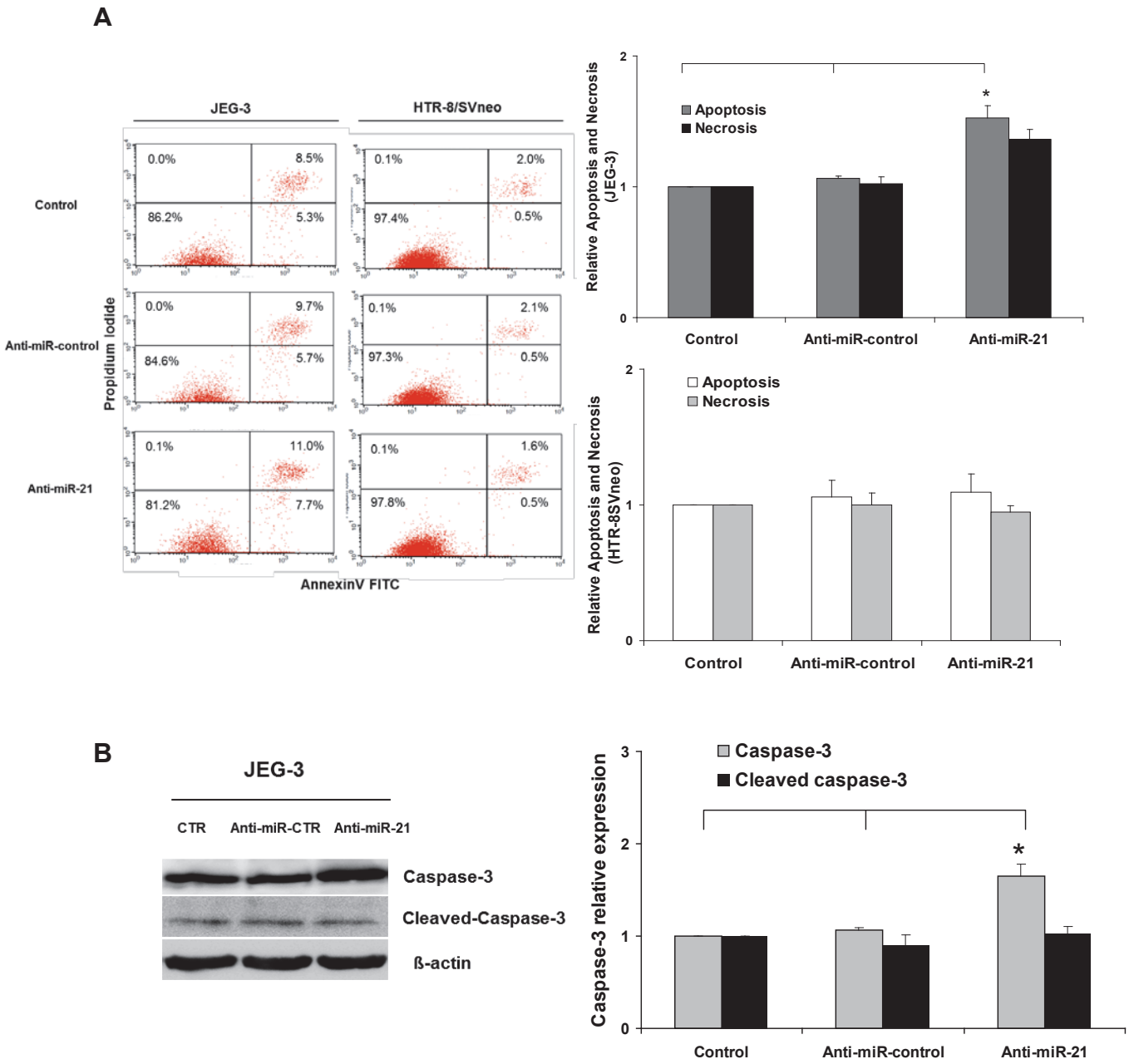
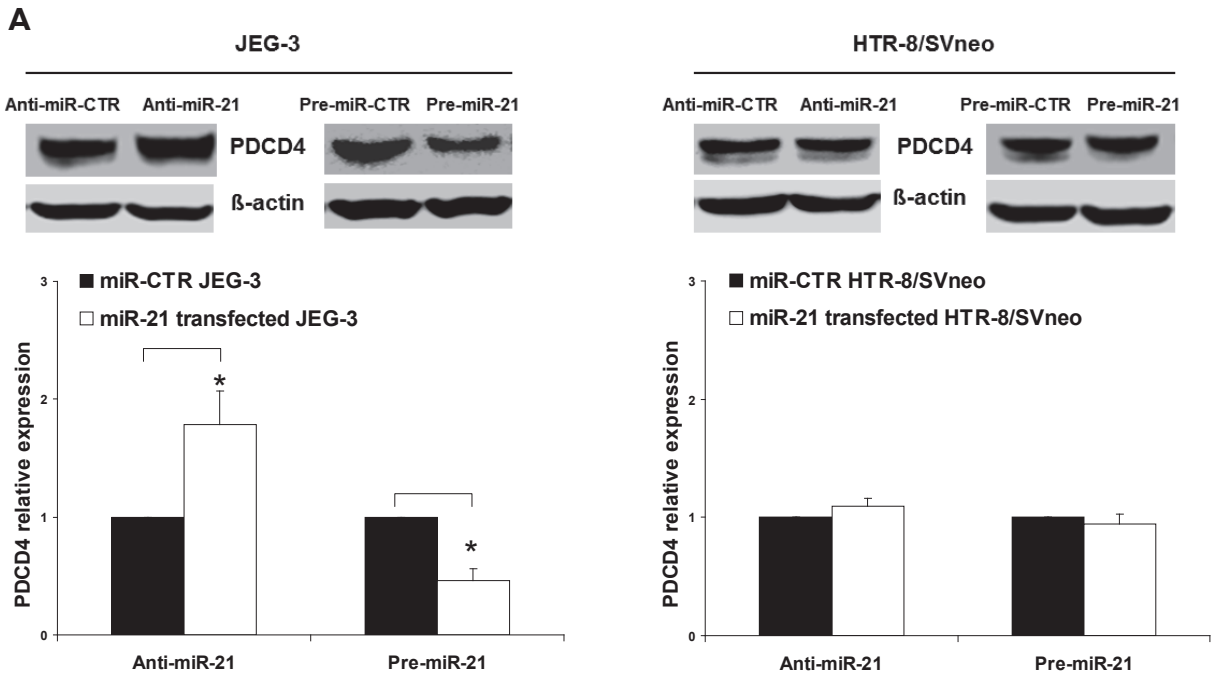
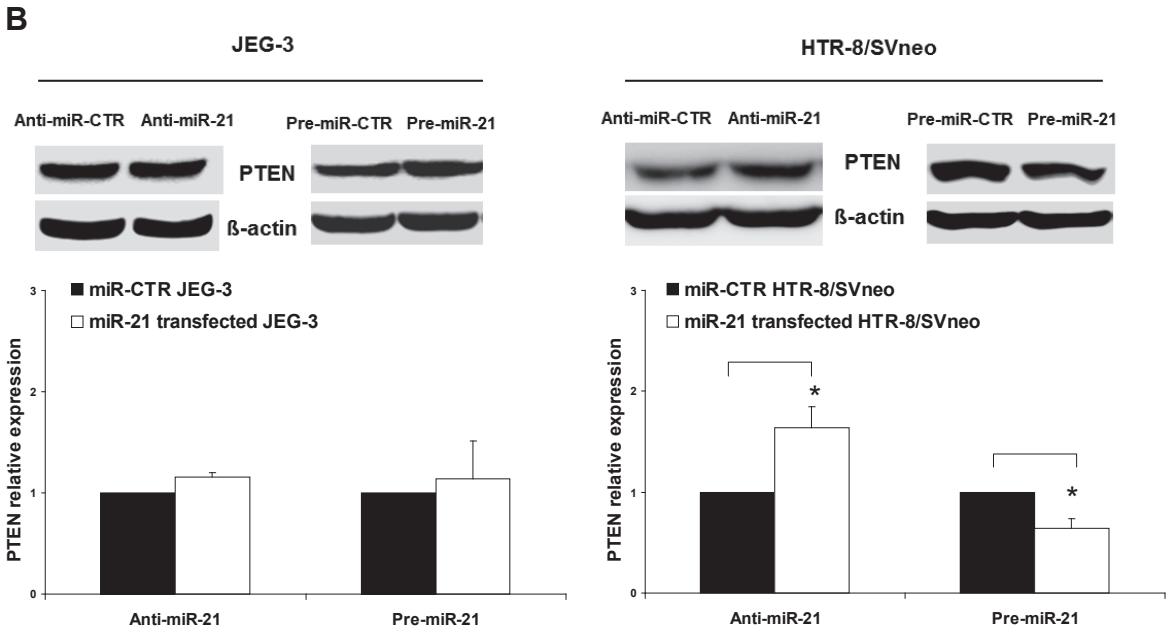

A
JEG-3
HTR-8/SVneo
Control
Anti-miR-control
Anti-miR-21
Propidium Iodide
AnnexinV FITC
0.0%
8.5%
86.2%
5.3%
0.1%
2.0%
97.4%
0.5%
0.0%
9.7%
84.6%
5.7%
0.1%
2.1%
97.3%
0.5%
0.1%
11.0%
81.2%
7.7%
0.1%
1.6%
97.8%
0.5%
Apoptosis
Necrosis
Relative Apoptosis and Necrosis (JEG-3)
Control
Anti-miR-control
Anti-miR-21
Relative Apoptosis and Necrosis (HTR-8SVneo)
B
JEG-3
CTR
Anti-miR-CTR
Anti-miR-21
Caspase-3
Cleaved-Caspase-3
ß-actin
Caspase-3
Cleaved caspase-3
Caspase-3 relative expression

Figure 5


A
JEG-3
HTR-8/SVneo
Anti-miR-CTR
Anti-miR-21
Pre-miR-CTR
Pre-miR-21
PDCD4
ß-actin
miR-CTR JEG-3
miR-21 transfected JEG-3
miR-CTR HTR-8/SVneo
miR-21 transfected HTR-8/SVneo
PDCD4 relative expression
Anti-miR-21
Pre-miR-21


B
JEG-3
HTR-8/SVneo
Anti-miR-CTR
Anti-miR-21
Pre-miR-CTR
Pre-miR-21
PTEN
ß-actin
miR-CTR JEG-3
miR-21 transfected JEG-3
miR-CTR HTR-8/SVneo
miR-21 transfected HTR-8/SVneo
PTEN relative expression
Anti-miR-21
Pre-miR-21

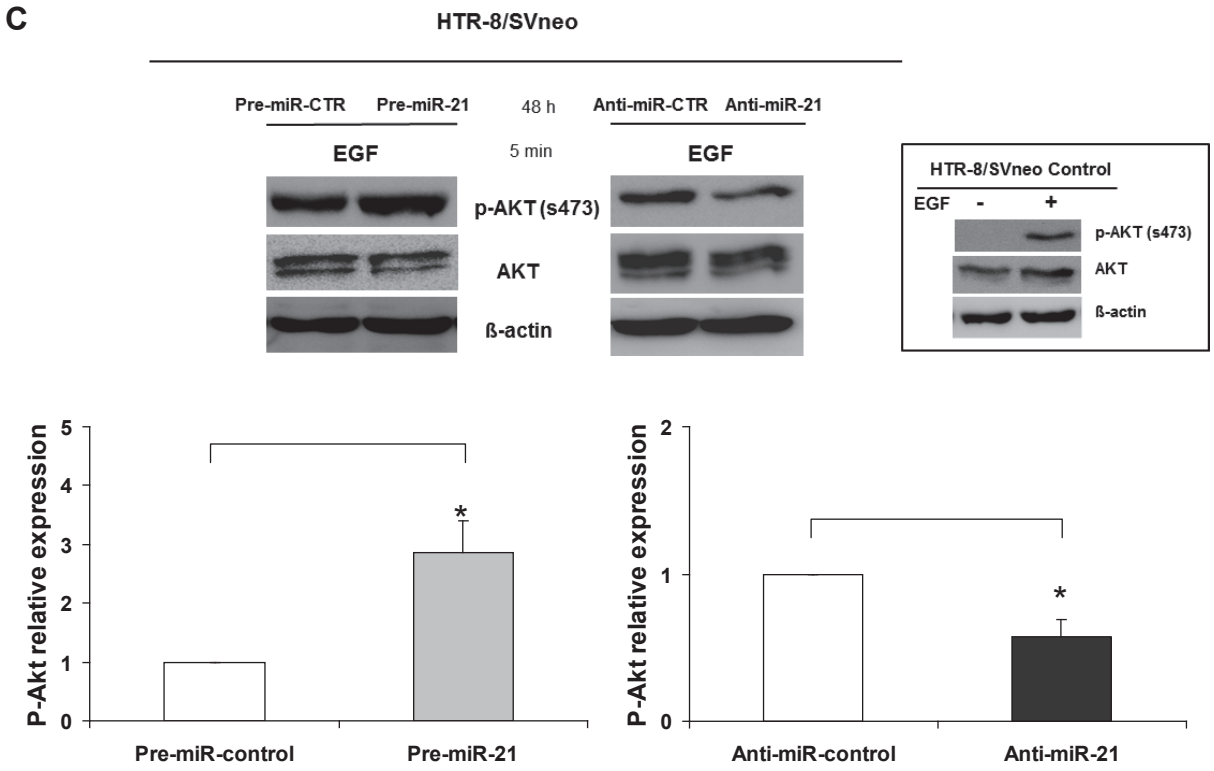
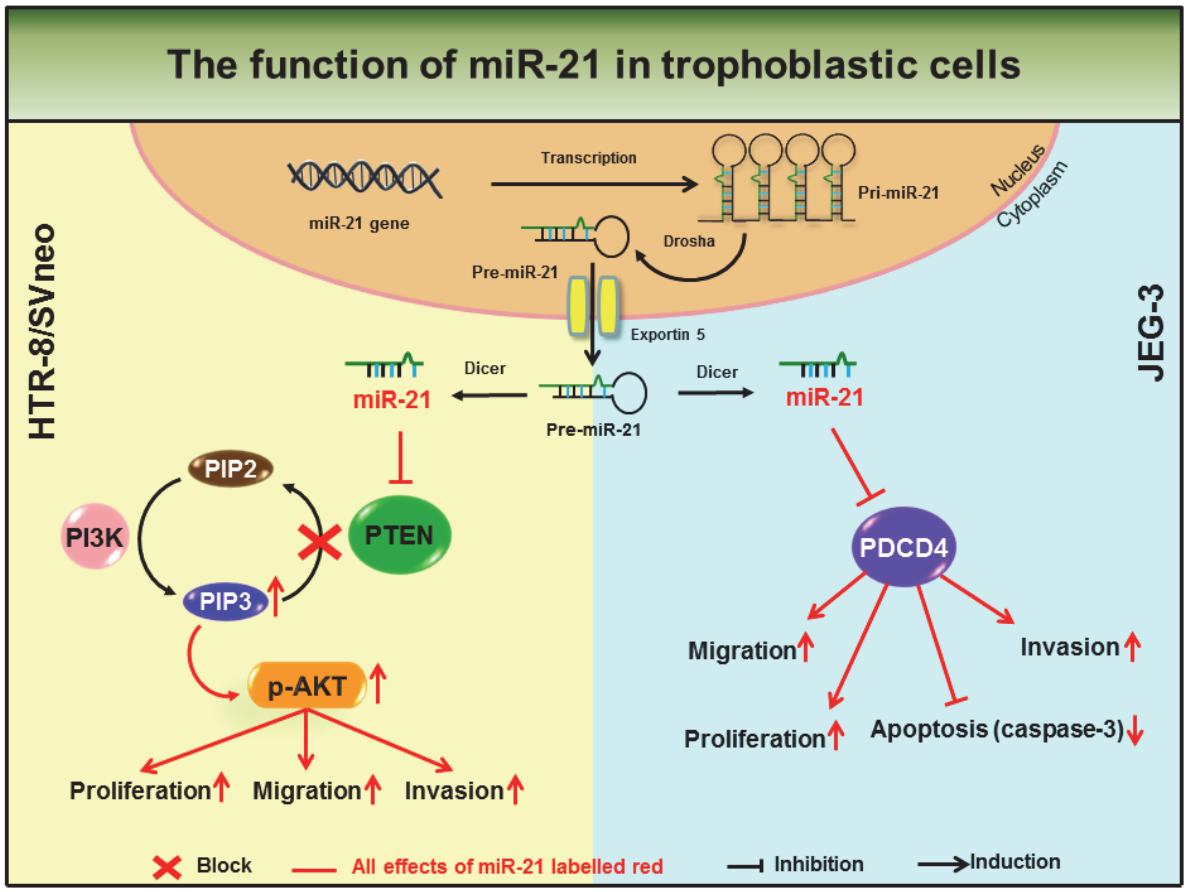

C
HTR-8/SVneo
Pre-miR-CTR
Pre-miR-21
48 h
Anti-miR-CTR
Anti-miR-21
EGF
5 min
EGF
p-AKT (s473)
AKT
ß-actin
HTR-8/SVneo Control
EGF - +
p-AKT (s473)
AKT
ß-actin
P-Akt relative expression
Pre-miR-control
Pre-miR-21
P-Akt relative expression
Anti-miR-control
Anti-miR-21

Figure 6


The function of miR-21 in trophoblastic cells
HTR-8/SVneo
JEG-3
Nucleus
Cytoplasm
Transcription
miR-21 gene
Pri-miR-21
Drosha
Pre-miR-21
Exportin 5
Dicer
miR-21
Pre-miR-21
Dicer
miR-21
PIP2
PI3K
PTEN
PIP3
p-AKT
Proliferation
Migration
Invasion
PDCD4
Migration
Invasion
Proliferation
Apoptosis (caspase-3)
Block
All effects of miR-21 labelled red
Inhibition
Induction

Chapter 5 Discussion

Human implantation is a crucial process for establishment of pregnancy. This process requires the coordinated functions of several hormones, cytokines and growth factors to be successful. Dysregulation of these factors can result in failure of implantation and termination of pregnancy. In the first publication of this thesis, the current knowledge of cytokines present at the implantation site and their influence on trophoblast cell functions has been reviewed (Fitzgerald et al. 2011). Among them, the IL-6 cytokine family plays an important role in human pregnancy. LIF, a member of the IL-6 family, has been most intensively studied in normal and abnormal conditions because of its essential function for blastocyst implantation.

LIF, LIFR and gp130 knockout mice have been intensively investigated. In LIF-deficient mice, failure of blastocyst implantation is often observed (Stewart et al. 1992). Interestingly, after infusion of LIF into the uterine lumen of these mice, the blastocyst can attach to the uterus resulting in normal pregnancy (Stewart et al. 1992). Lack of LIFR can result in normal implantation but with development of placenta dysfunction (Ware et al. 1995). Likewise, disruption of gp-130 gene causes embryonic lethality between day 12.5 and day 18 of gestation (Akira et al. 1995). In humans, LIF has been detected in uterine flushings from fertile women during time of expected implantation and in women with unexplained infertility LIF concentration is lower (Laird et al. 1997). LIF is expressed in endometrium and blastocyst during pre-implantation, and it is highest expressed in endometrium during implantation (Bhatt et al. 1991). In addition, LIF and its receptor are expressed by villous and extravillous trophoblast cells throughout pregnancy (Sharkey et al. 1999). OSM is a member of the IL-6 cytokine family and plays an important role in placental endocrine functions by inducing HCG release (Ogata et al. 2000). OSM concentration is significant higher in serum of pregnant women than in non-pregnant women (Ogata et al. 2000). Additionally, OSM stimulates trophoblastic cell proliferation and migration through STAT3 activation (Ko et al. 2013). In humans, OSM shares the gp130 receptor chain with LIF, (Richards 2013). Thus, it may be expected that LIF and OSM exhibit similar biological functions in trophoblastic cells. We have recently shown that at low

concentrations neither LIF nor OSM (10 ng/ml) has an effect on trophoblast cell proliferation, whereas, only LIF significantly increases trophoblast cell invasion (Chaiwangyen et al. 2014). Higher concentrations of OSM (20 ng/ml) have been shown to increase trophoblast cell proliferation, migration and invasion (Ko et al. 2012, Ko et al. 2013). These findings demonstrate that LIF and OSM are essential for blastocyst implantation and placental development potentially by controlling trophoblast functions. We have reported that, probably due to sharing their receptors, LIF and OSM have the capacity to activate STAT3 and ERK1/2 phosphorylation at different intensities (Chaiwangyen et al. 2014). Previously, our group has shown that LIF induces trophoblast cell invasion via STAT3 activation, while ERK1/2 activation is responsible for trophoblastic cell proliferation (Poehlmann et al. 2005, Prakash et al. 2011). The process of trophoblast cell invasion into decidual stroma is regulated by the activities of MMP-2 and MMP-9 (Pollheimer et al. 2014, Librach et al. 1991, Campbell et al. 2003, Shimonovitz et al. 1994). Our study demonstrates that LIF mediates trophoblast cell invasion by increasing MMP-2 and MMP-9 secretion through STAT3 activation (Chaiwangyen et al. 2014). Altogether, LIF shares JAK/STAT signaling with OSM but triggers different biological effects.

It is well documented that miRNAs are expressed in placenta tissues, trophoblast cells and maternal blood (Morales-Prieto et al. 2014, Morales-Prieto et al. 2012, Xie et al. 2014, Sarker et al. 2014). Therefore, most miRNAs may have the potential of serving as biomarkers. Our previous studies demonstrate changes of miRNA expression in trophoblast cells upon LIF stimulation (Morales-Prieto et al. 2011). Therefore, based on these findings and existing literature we aimed to investigate miRNA expression in different trophoblastic cell lines and primary trophoblast cells. Our result is consistent with the finding of Donker *et al* that placenta abundantly expresses miRNAs belonging to the clusters C19MC, C14MC and miR-371-3 (Morales-Prieto et al. 2012, Donker et al. 2012). C19MC is the largest human miRNA gene cluster. It is also highly expressed in other tissues, for instance embryonic stem cells and testis (Morales-Prieto et al. 2013, Ouyang et al. 2014). C14MC is conserved in mammals including mouse (Morales-Prieto et al. 2013, Ouyang et al. 2014). The miR-371-3 cluster is conserved in mammals and highly expressed in embryonic stem cells (Morales-Prieto et al. 2013). The level of C19MC expression

increases from first trimester placenta to third trimester placenta, whereas the expression of C14MC decreases from first trimester placenta to third trimester placenta (Morales-Prieto et al. 2012). Consistently with isolated trophoblast cells, C19MC miRNAs are highly expressed in JEG-3 and BeWo choriocarcinoma cells (Morales-Prieto et al. 2012, Luo et al. 2009), while HTR-8/SVneo cells (immortalized first trimester trophoblast) predominately express C14MC miRNAs (Morales-Prieto et al. 2012). These findings may be responsible for differences in proliferation and invasive capabilities of these cell lines. These results suggest that C19MC, C14MC and miR-371-3 miRNAs have important regulatory roles on trophoblast cell functions and placental development throughout pregnancy. Interestingly, our experiments reveal that miR-21 is one of the highest expressed miRNA in first trimester trophoblast cells (Morales-Prieto et al. 2012).

Individual miRNAs have been analyzed to discover their tissue specificity as well as their regulatory functions. So far, a limited number of miRNAs have been investigated in regard to their physiological and pathological functions in pregnancy. Recent studies demonstrated that dysregulation of more than 50 miRNAs are associated with preeclampsia by comparison of miRNAs expression profiles between preeclamptic- and normal placentas (Lee et al. 2011, Fu et al. 2013b, Luo et al. 2012, Bai et al. 2012, Hu et al. 2009, Zhu et al. 2009, Pineles et al. 2007, Ishibashi et al. 2012, Zhang et al. 2010, Wang et al. 2012b). Some of these miRNAs have been analyzed for their functional roles associated with preeclampsia including shallow invasion of trophoblast cells, reduced trophoblast proliferation and deficient maternal spiral artery remodeling. For example, miR-210 is up-regulated in preeclamptic placentas which display reduced trophoblast cell invasion (Enquobahrie et al. 2011, Pineles et al. 2007, Zhu et al. 2009, Zhang et al. 2012). Up-regulated miR-16 is associated with severe preeclampsia. Overexpression of miR-16 inhibits trophoblast cell migration and formation of tube like structures in HUVEC cells (Wang et al. 2012c).

Although miRNAs are produced by placenta and have regulatory functions on trophoblast cells (Table 1) it can be argued that not only placenta specific miRNAs regulate placental development and functions. It has been previously reported that elevated miR-21 expression is most common in all major classes of human cancers,

and therefore, known as oncomiR (Buscaglia und Li 2011). Moreover, miR-21 has been shown to enhance cell growth and to inhibit apoptosis in several cell culture and animal models (Li et al. 2012, Lu et al. 2014). MiR-21 is not belonging to placenta specific miRNAs but we have demonstrated that miR-21 is one of the highest expressed miRNA in first trimester trophoblast cells (Morales-Prieto et al. 2012). The function of miR-21 in trophoblastic cells and placental development has been largely unknown. In our studies, we demonstrated that the expression of endogenous miR-21 is higher in HTR-8/SVneo than in JEG-3 choriocarcinoma cells. The expression of miR-21 is positively correlated with proliferation, invasion and migration in HTR-8/SVneo and JEG-3 cells, which seems to be in line with previous reports that down-regulated miR-21 is associated with IUGR and preeclampsia (Maccani et al. 2011, Choi et al. 2013). These results suggest a leading role of miR-21 in regulation of trophoblast cell functions.

To understand the role of miRNAs in biological processes, it is essential to experimentally assess the functional relevance of predicted miRNA target sites. In general, miRNAs inhibit their target mRNA expression. Accordingly, a significantly negative correlation between miRNA and respective potential target mRNA confirms this interaction. PTEN and PDCD4 are tumor suppressor genes and have been validated as targets of miR-21 which are implicated in cell proliferation, migration, invasion and apoptosis in several cell types. (Buscaglia und Li 2011, Li et al. 2014a, Blanco-Aparicio et al. 2007, Lankat-Buttgereit und Goke 2009). In our results, we show that miR-21 targets PDCD4 in JEG-3 cells, whereas it targets PTEN/AKT in HTR-8/SVneo cells. Notably, $PTEN^{-/-}$ knockout mice have high embryonic lethality. These results indicate that PTEN is essential for early embryonic development (Di Cristofano et al. 1998, Suzuki et al. 1998, Freeman et al. 2006). In addition, PTEN plays an important role in implantation and is required for trophoblast invasion. Deletion of PTEN gene in mice leads to increased fetal mortality, abnormal development of the placental labyrinth, and IUGR (Lague et al. 2010). Furthermore, miR-21 is highly expressed at the implantation site which is regulated by the active blastocyst (Hu et al. 2008). Our current study demonstrates that miR-21 is involved in regulation of PTEN expression, probably mainly in first trimester trophoblast. Thus, our results highlight the expression of miR-21 and its targets, PTEN in HTR-8/SVneo

and PDCD4 in JEG-3 cells, which regulate trophoblast cell proliferation, migration, invasion and apoptosis during placental development. Thus, cell specific functions of miR-21 are likely to differ in different cell types. The target genes and signaling pathways of miRNAs that mediate trophoblast cell functions are still marginally understood and need further intensive studies both *in vitro* and *ex vivo.*

Several studies indicate that hypoxia may have an important impact during early placental development (8-10 weeks of gestation) by causing plugs of maternal spiral arteries by EVT cells (Figure 11) (Genbacev et al. 1997, Burton et al. 2002, James et al. 2006, Patel et al. 2010). Hypoxia inducible factors (HIFs) are transcriptional factors that mediate the cellular response to low oxygen levels and are involved in placental vascularization, trophoblast invasion and differentiation (Genbacev et al. 1997, Pringle et al. 2010, Doridot et al. 2013). More interestingly, miR-21 is one of hypoxamiRs which can regulate the expression of HIF1α in hypoxic stress (Nallamshetty et al. 2013, Kulshreshtha et al. 2007, Gorospe et al. 2011). Moreover, HIF1α regulates miR-21 expression by binding to hypoxia response element of miR-21 (Mace et al. 2013). These results strongly support our hypothesis that miR-21 regulates trophoblast cell functions during first trimester pregnancy. Prolonged fetal hypoxia is associated with IUGR and discovery of hypoxia-induced miRNAs in the maternal circulation has indicated its potential for becoming an IUGR biomarker (Bamfo und Odibo 2011, Regnault et al. 2007). It has been reported that miR-21 is highly expressed in maternal blood of severe FGR when compared to normal pregnancy (Whitehead et al. 2013) and placentas of IUGR and PE (Cindrova-Davies et al. 2013). Additionally, hypoxic conditions induce miR-21 expression in villous explants (Cindrova-Davies et al. 2013).

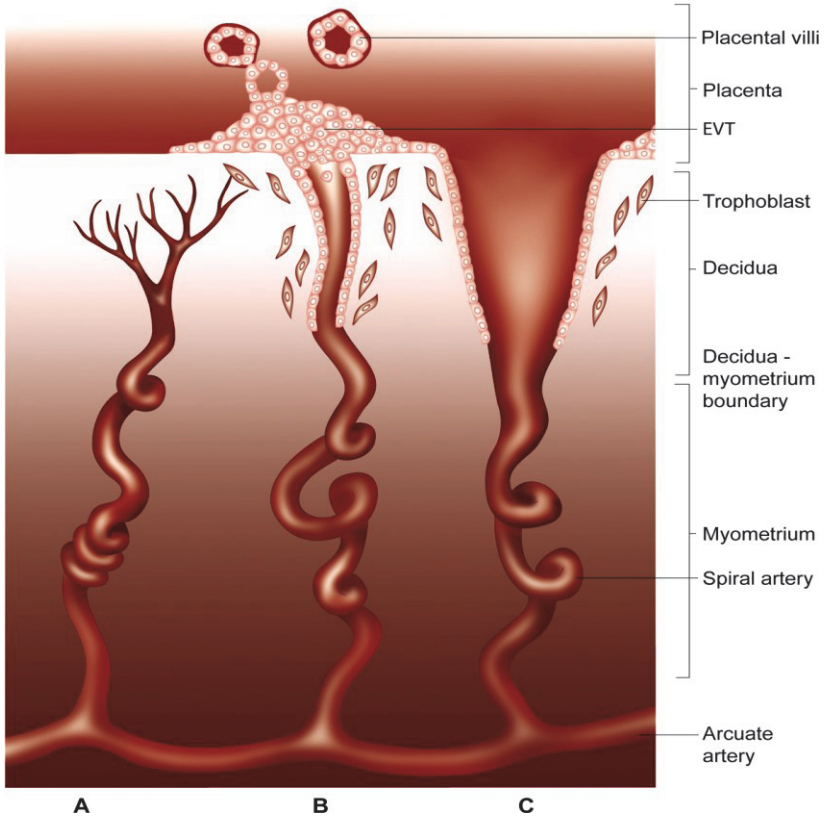


Figure 11. Extravillous trophoblast (EVT) cells block uterine spiral arterioles during first trimester pregnancy. A) Spiral artery before pregnancy, which forms a capillary plexus below uterine surface. B) At early first trimester pregnancy, endovascular EVT cells plug spiral arteries leading to low oxygen tension which is important for placenta development. C) Endovascular EVT cells remodel spiral arteries resulting in increased vascular diameter, blood flow and oxygen supply. Taken from Patel *et al* (Patel et al. 2010)

miRNAs have been detected in extracellular fluids such as serum or plasma (Mitchell et al. 2008, Luque et al. 2014). MiRNAs are packed and released from many tissue/cell types into extracellular compartments via microparticles (exosomes, microvesicles, apoptotic bodies) (Valadi et al. 2007, Zernecke et al. 2009). Circulating miRNAs are factors of long distance cell-cell communication and have emerged as powerful biomarkers for human diseases (Chen et al. 2012, Turchinovich et al. 2013, Creemers et al. 2012, Kosaka et al. 2013). miRNAs of the C19MC cluster are secreted into maternal circulation via exosomes which are highly expressed throughout gestation (Donker et al. 2012, Richardson 1990). The function of extracellular C19MC miRNAs includes the protection of the embryo from viral infection (Mouillet et al. 2014, Ouyang et al. 2014, Bullerdiek und Flor 2012). It has

been demonstrated that up-regulated miR-210 in serum of pregnant women is significantly associated with preeclampsia (Anton et al. 2013). Moreover, miR-210 levels can predict the progression of hypertensive disorders of pregnancy (HDPs) several months before the onset of symptoms (Anton et al. 2013). Thus, high levels of circulating miR-210 in pregnant women have a potential for becoming biomarker for prediction and diagnosis of preeclampsia.

Double stranded miRNAs or miRNA mimics containing the same sequence as the mature endogenous miRNA have been used to replace specific miRNAs that are down-regulated in diseases. Injection of Let-7 mimic can reduce tumor burden in a mouse xenograft model (Trang et al. 2010). Mir-34 is a tumor suppressor miRNA which inhibits cell proliferation and increases cell apoptosis. Synthetic miR-34 mimic (MRX34) is applied in a phase I clinical trial for liver-based cancers which will be completed by the end of first quarter of 2015 (Kelnar et al. 2014, Bouchie 2013). On other hand, specific antisense oligonucleotides (antagomiRs) have been developed for reducing miRNA levels which are up-regulated in pathological conditions. For example, Miravirsen which is antogomiR-122 reduces viral RNA with no evidence of resistance in hepatitis C (Janssen et al. 2013). Currently, Miravirsen is evaluated in a phase 2 clinical trial for treatment of Hepatitis C virus (HCV) infection (Gebert et al. 2014). Side effects, drug target delivery, and drug stability need to be further improved. The next challenge will be the development of strategies in order to increase or decrease specific miRNA levels in placenta aiming to prevent the progression of pregnancy related diseases.

Chapter 6 Conclusion

Cytokines and miRNAs play an important role during implantation and successful pregnancy. Our findings suggest that LIF regulates trophoblast cell invasion through MMP-2 and MMP-9 activation. Low concentrations of OSM have no effects on biological activity of trophoblast cells but its intracellular signals have some similarities with those induced by LIF, potentially activated through the gp130 receptor subunits. OSM has been detected at higher concentrations in serum of preeclamptic women than in normal pregnant women but further studies are required to confirm the relationship between OSM and preeclampsia. The role of OSM and LIF in cell proliferation and invasion seems to be cell type dependent. There are several trophoblast cell line models that were generated from different sources such as normal placenta, choriocarcinoma tissues and embryonic carcinomas for determination of molecular mechanism of trophoblast cell functions such as proliferation, invasion and apoptosis (Ji et al. 2013). Therefore translation of observed OSM and LIF effects to primary cells should be carefully evaluated.

Overall, the human placenta expresses miRNAs, which play important roles during placental development: 1) different patterns of miRNAs are expressed during different gestational stages 2) miRNAs are involved in several trophoblast cell functions including proliferation, migration, invasion, apoptosis and angiogenesis 3) altered miRNAs expression is associated with pregnancy related disorders, such as preeclampsia (Fu et al. 2013a). miR-21 regulates trophoblast cell functions and may potentially serve as biomarker for pregnancy complications. Circulating miRNAs may be useful for clinical application as novel diagnostic markers or potential therapeutic targets.

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Ehrenwörtliche Erklärung

Hiermit erkläre ich, dass mir die Promotionsordnung der Medizinischen Fakultät der Friedrich-Schiller-Universität bekannt ist,

ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind,

mich folgende Personen bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt haben: Prof. Dr. med Udo Markert, Dr. Diana Morales-Prieto und Stephanie Ospina-Prieto.

die Hilfe eines Promotionsberaters nicht in Anspruch genommen wurde und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen,

dass ich die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe und

dass ich die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung nicht bei einer anderen Hochschule als Dissertation eingereicht habe.

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06.2013 33st Annual Meeting of the American Society of Reproductive Immunology. Boston, USA. Poster Presentation

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SCIENTIFIC PAPERS

- **Chaiwangyen W**, Ospina-Prieto S, Photini SM, Schleussner E, Markert UR, MoralesPrieto DM. miR-21 regulates trophoblast cell functions by targeting phosphatase and tensin homologue (PTEN) and programmed cell death 4 (PDCD4). *RNA Biol* (submitted)

- Ospina-Prieto S, **Chaiwangyen W**, Schleussner E, Markert UR, MoralesPrieto DM. MiR-141 is altered in preeclampsia tissues and regulates trophoblast invasion and intercellular communication. (to be submitted soon)

- Ospina-Prieto S, **Chaiwangyen W,** Pastuschek J, Schleussner E, Markert UR, Morales-Prieto DM. STAT 5 is activated by Epidermal Growth Factor (EGF) and induced proliferation and invasion in trophoblastic cells. *Repro Sci.* 2015 April 9

- **Chaiwangyen W**, Ospina-Prieto S, Morales-Prieto DM, Pereira De Sousa FL, Pastuschek J, Fitzgerald JS, Schleussner E, Markert UR. Oncostatin M and leukaemia inhibitory factor trigger signal transducer and activator of transcription 3 and extracellular signal-regulated kinase 1/2 pathways but

result in heterogeneous cellular responses in trophoblast cells. *Reprod Fertil Dev*. 2014 Sep 24.

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